CHARACTERIZATION OF CONNECTIVE TISSUE OF BOVINE SKELETAL MUSCLES AND THERMAL AND CHEMICAL MODIFICATION OF EPIMYSIUM TO DECREASE SHEAR STRESS

A Thesis Submitted

to the College of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Food and Bioproduct Sciences

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

Anula Perera

2009

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may take it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purpose may be granted by the professor who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Food and Bioproduct Sciences

University of Saskatchewan

51 Campus Drive

Saskatoon, SK

Canada, S7N 5A8

ABSTRACT

This research was conducted to investigate the connective tissue contribution to toughness of cow beef and to find means to decrease it. Intra muscular connective tissue (IMCT) content of meat from cows (~6 years) and heifers (~16 months) varied significantly among muscles ($P<0.0001$) and maturity groups ($P<0.05$). Amount of total collagen in IMCT was a constant (37.3-46.3 %) among muscles and between maturity groups. Shear force of *biceps femoris, semimembranosus* and *longissimus* muscles had increased significantly with animal maturity ($P<0.0001$). Shear stress of *gluteus medius* was similar between maturity groups. Collagen solubility decreased with animal maturity, except for *biceps femoris*.

The impact of the temperature of aqueous heating (55 to 95 ºC) and time on thermolabile proteins, amorphous proteins, Ehrlich chromogen, pyridinoline, thickness change, shrinkage, weight gain, shear force, amide bands and morphology of epimysium was studied. Collagen contributed to 90% (w/w) of epimysial proteins. At 55 ºC, epimysial properties were changed only after exposure to long heating times. Shear stress values of raw cow (39.6 N/mm^2) and heifer (30.8 N/mm^2) epimysium decreased significantly to 11.6 and 2.1 N/mm^2, respectively, at 70 ºC. Amount of epimysial amorphous collagen (14-16% w/w) detected after heating at 70 ºC and above was not related to shear stress decrease. Before and after heating, cow epimysium contained more pyridinoline cross-links than heifer epimysium.

The effects of strong and weak acids and alkalis on epimysial properties were studied following heating at 55 and 70 ºC for 15 min. As the concentration of HCl (0.1-0.5 M) and pre-equilibration time were increased at 70 ºC, shear stress decreased to <2 N/mm^2. Increasing concentration of CH_3COOH (0.1-0.5 M) and pre-equilibration times had decreased shear stress to ~5 N/mm^2. At 55 ºC, HCl was not superior to CH_3COOH in its ability to decrease epimysial shear stress. Increasing concentration of NaOH (0.01-0.05 M) and high temperature decreased

shear stress to ~3 N/mm^2. Lack of a shear stress decrease at 55 ºC and increased thermal denaturation temperature (66 ºC compared to 63 ºC in water), indicated that NH_4OH had an epimysial stabilization effect, which was not eliminated at 55 ºC.

ACKNOWLEDGEMENTS

I express my gratitude to Dr. Phyllis J. Shand, my supervisor, for guiding me, for investing immense amount of her time on my manuscript and supporting me in numerous ways through out the course of this study.

I gratefully acknowledge the valuable advice and time contributions of the members of the advisory committee, Dr P. J. Shand, Dr. D. R. Korber, Dr. Z. Pietrasik, Dr. M. Nickerson and Dr. A. Olkowski. I also acknowledge the valuable suggestions and time contribution from the external examiner, Dr R. Miller, Texas A & M University. I also extend my special thanks to Dr. A. Olkowski for providing me with training on Scanning Electron Microscopy and also for conducting FTIR analysis. I extend my thanks to Dr. Z. Pietrasik for his immense support and advice throughout this study. I am grateful to Dr J. Lepetit of Institut National De La Recherche Agronomique, France for his valuable comments on my manuscript and suggestions.

My sincere thanks are extended to Dr. M. Pato, of the Department of Biochemistry for giving access to her laboratory all through out this study. Also, my thanks are extended to Dr. J. Wanasundara, of Agriculture and Agrifood Canada, Saskatoon for providing access to the Differential Scanning Calorimeter in her laboratory and Ms Sara Lui, of Saskatchewan Food Industry Development Centre Inc. for giving access to her laboratory to carry out preliminary studies. I acknowledge the transmission electron microscopy services provided by the Advanced Microscopy Facility of the Department of Biological Sciences, University of Alberta.

I sincerely acknowledge the technical assistance of H. Silcox, N. Weibe and D. Pobereznek of the Department of Food and Bioproduct Sciences, University of Saskatchewan during this study. I am also thankful to T. Bonley of Geological Sciences for his assistance during Scanning Electron Microscopy work, A. Givens of the Department of Biochemistry for her assistance

during electrophoresis work, T. MacIntosh of Agriculture and Agrifood Canada, Saskatoon and A. Opoku of the Department of Agricultural and Bioresource Engineering, University of Saskatchewan, for their assistance during Differential Scanning Calorimetric work. I also acknowledge the Canadian Light Source, NSERC, NRC, CIHR, and the University of Saskatchewan for supporting FTIR work. I express my thanks to Canadian Cattlemen's Association, Agriculture Development Fund of the Saskatchewan Ministry of Agriculture, University of Saskatchewan Devolved Scholarship, Rene Vandeveld Post Graduate Scholarship and my supervisor Dr Phyllis Shand, for the financial assistance provided to complete this research.

I express my sincere appreciation to my husband and my daughter for being my strength and for their love, patience, empathy and emotional support extended to me during my ups and downs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Canada was the third largest exporter of beef in the world prior to the discovery of a single case of bovine spongiform encephalopathy (BSE) on May 20, 2003 and the subsequent worldwide ban of Canadian beef products. Prior to the ban, almost half of the cattle sold in Canada were exported as either live animals or meat and the total amounted to 3.4 million cattle (Poulin and Boame, 2003). In September 2003, the United States of America and several other countries had lifted the ban on import of boneless beef from animals younger than 30 months (Boame et al., 2004). Later in July 2005, US embargo on live cattle imports from Canada ended for cattle and bison less than 30 month of age. However, strict regulatory control is envisaged for implementation of standards, traceability, monitoring and testing. The outcome of the ban and restrictions was the challenge of selling mature cow and bull beef within Canada (Sparling and Caswell, 2006; LeBlanc, 2007; Rude et al., 2007). As such, the industry needs measures to mitigate the inherent quality problems associated with mature beef, including toughness, to exploit the Canadian retail market.

Toughness or its reciprocal tenderness is often considered as one of the key factors that determine consumer acceptability of meat (Lorenzen et al., 2003; Feuz et al., 2004). It was not surprising to understand that before the restriction of international trade of beef from mature animals, most of the beef tenderness research in North America was conducted on meat from quality grades originating from young animals (Morgan et al., 1991; Wheeler et al., 1999; Wheeler et al., 2002) because local consumers were then supplied with tender cuts. Despite the flooding of the local market with beef from mature animals subsequent to the ban, little research was conducted, before and after the ban, to compare beef from young and old animals (Reagan

et al., 1976; Shorthose and Harris, 1990; Xiong, 2007; Li et al., 2007). As such, there is a pressing need to understand the quality characteristics of beef from mature animals.

As understood today, beef toughness is a multifaceted problem. Genetics of the animal influences the toughness differences between breeds (Monsón et al., 2004). More prominently physiological variations among muscle groups (Torrescano et al., 2003), fibrosis during wound healing (Kisseleva and Brenner, 2008), post-mortem metabolism as influenced by ante-mortem conditions such as stress level before slaughter (Lahucky, et al., 1998), rigor development after death as influenced by conditions of handling (Hostetler, et al., 1970; Newbold and Harris, 1972) and physiological maturity of animals often designated as chronological age (Shorthose and Harris, 1990) have a great impact on tenderness/toughness differences observed between muscles and animals.

Toughness of beef is generally attributed to toughness originating from myofibrillar proteins and from connective tissues (Ouali, 1990; Purslow, 2005; Koohmaraie and Geesink, 2006; Lepetit, 2007). Total collagen content is often considered as an indictor of connective tissue content in meat and a contributor to toughness (Hill, 1966; Burson and Hunt, 1986; Seideman, 1986) but contradictory findings also are reported (Maher et al., 2005; Delagdo et al., 2005). The contribution of connective tissue to toughness was mostly studied while connective tissue was still attached to the muscle matrix (Møller, 1981; Swatland, 2006) and the direct measurements on toughness of connective tissue were seldom made (Field et al., 1970). This thesis presents an investigation on the contribution of beef connective tissue to toughness and also thermal and chemical modification of connective tissue properties to decrease toughness.

Historically, heating is the best-known way to decrease toughness of meat. However, the effect of cooking temperature on myofibrillar and connective tissue proteins were not clearly

established due to the fact that heating was carried out while connective tissues were embedded in meat (Bouton and Harris, 1972; Davey and Gilbert, 1975; Christensen et al., 2000; Aktas, 2001; Brooks et al., 2004). Though the heating effect on major connective tissue protein collagen was investigated thoroughly (Wright and Humphrey, 2002), heating effect on meat connective tissues (matrix bound collagen) was scarcely studied. As such, gaps exist in the understanding of the thermal properties of meat connective tissue, which needs to be bridged.

Over the years, various mechanical, chemical and enzymic methods have been experimented to decrease the toughness of beef. Mechanical tenderization is designed to pierce meat tissue (Seideman et al., 1986; Loucks, et al., 1984; Pietrasik and Shand, 2004). Tenderization of beef with various enzymes such as papain, bromalin, ficin and ginger rhizome proteases has been thoroughly investigated (Tappel et al., 1956; Kang and Rice, 1970; Thompson et al., 1973; Kang and Warner, 1974; Fogle et al., 1982; Rolan et al., 1988). The chemical approach for tenderization included activation of natural muscle enzyme calpain through injection of calcium chloride during early post-mortem (Nagainis and Wolfe, 1982; Wheeler et al., 1997; Kong, et al., 2006), application of sodium bicarbonate (Anna et al., 2007), application of mixtures of various salts such as sodium and potassium di- and tri-phosphate, lactate and chloride (Hoffman, 2006) and application of acids such as lactic (Berge et al., 2001), acetic and citric (Arganosa and Marriot, 1989). Other chemicals tried in meat tenderization included sodium fluoride, sodium acetate and sodium citrate (Jerez et al., 2003). Because all these mechanical, enzymic and chemical treatments were applied on meat in which both myofibrillar and connective tissue proteins were present as one unit, it was difficult to conclude the treatment effect on individual components; in other words, the reason for the decreased toughness was not clear. The effects of acids and alkali treatments on isolated collagen was well researched (Knaggs, 1929; Theis and Jacoby, 1943; Bowes and Kenten, 1950), their effect on meat connective tissue (matrix bound collagen) and also their contribution to connective tissue driven toughness were not

investigated. Thus, it was thought important to assess the impact of selected treatments on connective tissue to better understand the influence of this muscle component.

In general, connective tissue driven toughness of beef is a problem yet unresolved and requiring further exploration, not only to find means to decrease toughness but also to understand its underline causes.

Study I

Hypothesis: Intramuscular connective tissue content in a muscle is an indicator of beef toughness.

Objectives: To characterize selected bovine muscles on intramuscular connective tissue properties such as quantity of connective tissue, total collagen, soluble collagen, thermal transition temperatures of muscle proteins and to establish a relationship between tenderness and measured parameters.

Study II

Hypothesis: The degree of conversion of collagen from native to denatured or amorphous state determines the degree of connective tissue driven toughness.

Objectives: To investigate the effect of temperature (corresponding to the beginning and the end of phase transition of collagen) and the duration of heating on the epimysial shear stress, thickness change, weight gain, protein release, production of epimysial amorphous collagen, pyridinoline cross-links and also ehrlich chromogen content in amorphous collagen. Also, this study investigated the morphological changes of epimysium subsequent to heating.

Study III

Hypothesis: (a) A strong proton donor has an increased capacity to weaken the epimysium structure than a weak proton donor through the hydrolysis of bonds of the collagen and (b) a strong alkali has an increased capacity to weaken the epimysium structure than an alkali that dissociates weakly.

Objectives: To dissociate strong inter molecular bonds of cow epimysium using acids and alkali and measure thickness change, weight gain, quantity and quality of protein release and amino acids release as indicators of weakened structural integrity.

2. LITERATURE REVIEW

2.1 Bovine skeletal muscles

The main function of skeletal muscle in living animals is to provide power for locomotion. This is facilitated by the muscle fiber architecture, the arrangement of fibres relative to the axis of force generation. Accordingly, muscles have a longitudinal architecture where fibres arrange along the axis of force generation, unipennate architecture with a fixed angle of fibre arrangement relative to the force-generating axis and multipennate architecture with multiple angles of fibre arrangement relative to the force-generating axis (Lieber, 1992).

Commercially important skeletal muscles of bovine carcass are grouped as (a) neck muscles, where *sternomandibularis* is the only significant muscle and the rest are mechanically removed for ground beef, (b) shoulder muscles which include *supraspinatus*, *infraspinatus*, *biceps brachii* and *triceps brachii*, (c) ribcage muscles consisting of *serratus ventralis*, *longissimus dorsi* a compound muscle spreads over the length of several vertebrae, *pectoralis*, *intercostales externi* and *intercostales interni*, (d) loin muscles including *longissimus dorsi*, *psoas major*, *psoas minor* and *longissimus costarum*, (e) hind limb muscles including *gracilis*, *sartorius*, *quadriceps femoris*, *vastus medialis*, *vastus lateralis*, *vastus intermedius*, *rectus femoris*, *semitendinosus*, *semimembranosus*, *adductor*, *pectineus*, *biceps femoris* and *gastrocnemius*, (f) sirloin muscles consisting of *gluteus medius*, *gluteus accessories* and *gluteus profundus*, and (g) flank muscles formed by sheets of muscle and connective tissue (Swatland, 1994). As per the general classification, neck and shoulder muscles are located in the chuck region, rib cage, sirloin and loin muscles are sited in rib and sirloin/loin area and hind limb muscles are observed in hip area of the beef carcass (Figure 2.1). Different methods of carcass fabrication are

reported in the literature, including the conventional approach, the 'innovative cut' technique (Pfeiffer et al., 2005) and hot boning (Seideman and Cross, 1982). Conventional carcass fabrication includes separation of fore- and hindquarters followed by separation of rib, chuck and brisket (and so on) by saw cutting. Meat from the cuts is then separated after trimming off fat. Briefly, the innovative cut fabrication process includes, in sequence, separation of fore- and hindquarters of carcasses, trimming off all major connective tissues and fat and finally separation of individual muscles of each quarter from their points of attachment (Pfeiffer et al., 2005). In hot boning, lean meat and fat are removed from the bones prior to chilling (Seideman and Cross, 1982; Wheeler et al., 1991).

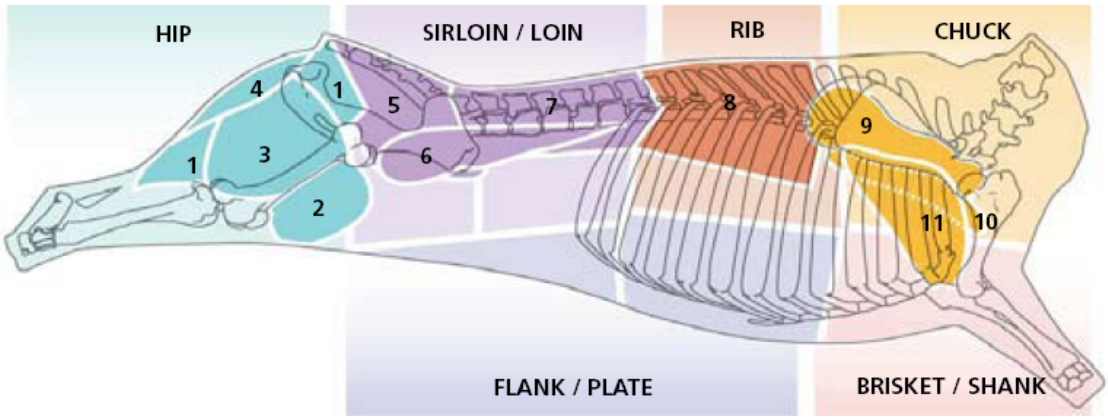


Figure 2.1 Distribution of commercially important muscles in a beef carcass. Hip area comprises of muscles, 1. *Biceps femoris*, 2. *Rectus femoris*, 3. *Semimembranosus* and 4. *Semitendinosus*. Loin and rib region contains muscles, 5. *Gluteus medius*, 6. *Psoas major*, 7. *Longissimus lumborum* and 8. *Longissimus thoracis*. Chuck of the carcass includes, 9. *Infraspinatus*, 10. *Teres major* and 11. *Triceps brachi*. Figure from Commercial Beef New Markets reproduced with kind permission from The Beef Information Centre, Calgary AB Canada www.canadianbeef.info

2.2 Myofibrillar proteins

Skeletal muscles serve as the major repository of protein in the living animal and account for about 50% of body proteins and amino acids. Following slaughter, when muscles convert to meat through biochemical changes muscle proteins becomes a source of nutrition (Rooyackers and Nair, 1997). Generally, skeletal muscles contain about 19% protein and from that about 11.5% are myofibrillar proteins, about 5.5% is sarcoplasmic proteins and about 2% are stromal proteins. Myofibrillar proteins consist of myosin, actin (monomers and fibres), tropomyosin, troponins, α-actinin and other minor proteins (Findlay and Barbut, 1990), which participate in muscle contraction.

Myosin is a family of major contractile proteins in muscle. Purified myosin consists of two globular heads (also called S1 region) joined by α-helical, coiled-coil tail. Tails of myosin interacts with each other to form a bipolar filament. Each head has an actin-binding site and shows actin–activated ATPase activity. Each myosin molecule consists of three different gene products, a heavy chain (230 kDa) and two light chains (20 kDa each) (Warrick and Spudich, 1987) where light chains are not covalently bound. Myosin is insoluble in low ionic strength solution and forms aggregates. To increase the solubility for research purposes, myosin is digested with chymotrypsin (Chalovich, 1992).

Globular actin monomers are arranged in helical strands. Two actin strands are twisted about each other with cross over at every 350-380 Å to form an actin molecule. There are about 13 actin monomers between two crossovers (Chalovich, 1992; Geeves and Holmes, 1999). α-Actinin is observed to bind on to a single actin filament at crossover points. It was proposed that, binding of α-actinin sterically blocks other protein binding on to actin and anchor actin filaments to adhesion proteins (Hampton et al., 2007). Troponin regulates skeletal muscle contraction through three non- identical protein subunits; (a) TnC with a molecular weight of

17.85 kDa binds Ca^{2+} at both amino and carboxyl terminal ends of the peptide to facilitate actomyosin ATPase activity, (b) TnI with a molecular weight of 20.86 kDa inhibits actomyosin ATPase activity and (c) TnT with a molecular weight of 30.50 kDa binds the molecule to actin filament and regulates muscle contraction (Zot and Potter, 1987). Tropomyosin has a molecular weight of 90.50 kDa (Bailey, 1948) and regulates muscle contraction. Tropomyosin consists of two parallel α-helical polypeptide chains, which are linked in coiled-coil fashion. This is flexible protein has the ability to twist when binds to actin (Weber and Murray, 1973). The molecular theories and mechanisms associated with muscle contraction (Davies, 1963; Farah and Reinach, 1995; Geeves and Holmes, 1999) will not be discussed as it is out of the scope of this review.

2.3 Connective tissue in skeletal muscle

The major functions of connective tissue include allowing efficient movement, acting as an endoskeleton to protect and support soft tissues, participating in homeostasis through the mediation of fibroblasts to regulate extracellular matrix turnover during injury and pathology, energy storage through fat and polysaccharides including glycogen and thermal regulation through fat reserves (Meyer and Rapport, 1951; Weber, 1999; Freemont and Hoyland, 2007). Connective tissue in skeletal muscles contains three levels of organization (endomysium, perimysium and epimysium), which are morphologically different. Endomysium and perimysium are embedded in the muscle matrix and together referred to as intramuscular connective tissue (McCormick, 1994). The endomysium functions as myocyte-myocyte connectives, myocyte-capillary connectives and also as a weave of fibres covering individual myocytes. Perimysium consisted of tightly woven bundles of collagen fibres oriented either parallel to the muscle bundles or circumferential to muscle bundles. These perimysium bundles are sub-divided into smaller bundles to form a meshwork around groups of myocytes (Bourg and Cauldfield, 1980). Epimysium covers muscles but can be easily separated. Both

perimysium and epimysium facilitate vasculature and adipose deposits within muscles (McCormick, 1994).

Collagen from both perimysium and endomysium are arranged in a 'cross-ply' pattern (number of different layers of similar thickness but with alternating orientations of 0º-90º) and the fibres within a ply (layer) are arranged parallel to each other (Purslow, 1989). The mechanical properties of muscles at extension are influenced by the orientation of the perimysial collagen network. That is, collagen fibres of perimysium are laid at ±θ angle to the muscle fibres. Generally, θ ranges within 45º-60º but in extremely shortened muscles θ can increase up to 75º, with the possibility that the crimps of collagen become straightened out (Rowe, 1974). Endomysium is a membranous honeycomb structure consisted of tightly arranged collagen fibrils of 30-70 nm in diameter. Perimysium consists of several layers of thick collagen sheets surrounding the sheaths of endomysium (Nishimura, 1994; Tabata et al. 1999). According to scanning electron micrographs, perimysium has four levels of fibre organization closely linked with the function, (i) perimysial junctional plates that constitute the focal attachment between the perimysium and the myofibers, (ii) collagen fibres attaching adjacent myofibres, (iii) a loose lattice of large interwoven fibres, and (iv) honeycomb tubes connecting two tendons (Passerieux et al., 2006; 2007). Conversely, the latter description of perimysium is similar to the description given for endomysium by Bourg and Cauldfield (1980). The perimysial content of a skeletal muscle is several folds higher than the endomysial content of the same muscle (Light et al., 1984). Also, the perimysial collagen content extensively varies among bovine muscles but endomysial content does not vary significantly (Purslow, 1999). The differences in connective tissue content are believed to reflect the differences in force transmission abilities of muscles (Huijing, 1999; Trotter and Purslow, 1992).

The epimysium consists of two distinct layers of thick sheets; an inner layer with thousands of collagen fibers lying in an highly regular wavy pattern parallel to the axis of the muscle fiber and an outer layer with several wavy sheets of collagen fibers extended transversely to the muscle fiber axis. Epimysium also has about 1 mm thick region, where fiber bundles extend longitudinally and parallel to the muscle fiber axis (Nishimura, 1994).

2.4 Connective tissue proteins

Connective tissue consists of a heterogeneous mixture of macromolecules and provides mechanical strength to muscle, define boundaries of organs and also participates in intercellular signalling (Ramirez et al., 2007). Even though the extra cellular matrix is ubiquitous, its composition significantly varies from organ to organ. Extra cellular matrix proteins are secreted by fibrocytes, the extra cellular matrix cells, and consist of structural proteins collagen, proteoglycans, elastin, fibrillin and fibronectin (Williams et al., 1989). The biosynthesis of collagen, enzymatic and non-enzymatic cross-linking of collagen, collagen metabolism and other constituents of connective tissue such as proteoglycans, elastin, fibrillin and fibronectin are discussed in the following section.

2.4.1 Collagen

As Estoe (1967) stated, 'the English word collagen has its roots in the French word *collagène* where *colla* is derived from Greek *Κολλα* or glue and *gène* being taken in the sense of producing'. According to titration curves and water uptake patterns, the isoelectric points of collagen, gelatin and hydrolysed collagen are 8.26, 4.88 and 4.54, respectively (Bowes and Kenten, 1948; Zhang et al., 2006). The amino acid composition of acid and citrate soluble collagen and gelatine were extensively investigated (Bowes et al., 1955; Estoe, 1955; 1967). Glycine, the simplest amino acid, is the most abundant in collagen and accounts for one residue in every three residues of amino acids. The second simplest amino acid alanine is the next most

abundant and makes up for one in every nine residues of amino acids. Proline and hydroxyproline are the other major amino acids in collagen; proline accounts for less than one in every eight residues and hydroxyproline accounts for more than one in every eleven residues. Because glycine, alanine, proline and hydroxyproline account for two in every three residues, collagen is considered as a basic protein. The molecular conformation of the collagen peptides confers the strict amino acid sequence of *(Gly-X-Y)(n)*, a repeating pattern, where *Gly* is glycine and *X* and *Y* are (mostly) proline and hydroxyproline, respectively. Together, proline and hydroxyproline account for one third of the total amino acids in *X* and *Y* positions (Eyre, 1980; Brodsky and Persikov, 2005).

Ramachandran and Kartha (1954; 1955) proposed the structure of the collagen molecule. Accordingly, a collagen molecule has a triple helical configuration where three peptide chains are wound together. Each single chain has 'minor' helices containing 10 amino acids per 3 turns. Three such chains are wound to form a 'major' helix with a radius of 2.5 Å. Each turn in the major helix contains 30 amino acid residues. Thus, 30 residues make 9 turns in a single chain (Ramachandran and Kartha, 1955). The major helix is wound in a right hand direction and the minor helices are wound in a left hand direction.

The triple helical structure is stabilized through hydrogen bonds where two of every three NH groups are hydrogen bonded to an oxygen atom from a C=O group (Ramachandran and Kartha, 1955). Therefore, one oxygen atom from every three C=O groups is not hydrogen bonded within the helix and thought to be bonded to other helices around. Rich and Creek (1955) proposed an alternate theory with a collagen molecule stabilized with one hydrogen bond for every three residues. The two theories were amalgamated later and accordingly, the second hydrogen bond is formed through a water molecule which also is hydrogen bonded to OH groups of hydroxyproline (Ramachandran, 1988). An X-ray crystallographic study of a collagen

like synthetic peptide has confirmed the presence of inter- and intra-chain hydrogen bonding. Also, it confirms the presence of a hydration cylinder around collagen molecules with an extensive network of hydrogen bonding between water molecules and peptides stabilizing the structure (Bella et al., 1994). Further, this cylinder is formed by the water molecules in close contact with H-bond acceptor of collagen and prevents peptide exposure to bulk water (Figure 2.2). Crystallographic evidence further supports the theory of hydrogen bonding in collagen through two repetitive patterns of hydrogen bonds, 'weak C^{α}-H…O=C and strong N-H…O=C' bonds (Bella and Berman, 1996). The latter bond is thought to be responsible for the alignment of three chains. These weak inter chain hydrogen bonds (C^{α}-H…O=C) are located between α-carbon hydrogen from glycine and hydroxyproline residues and C=O groups from glycine and proline residues of neighbouring chains. Not all agree on the concept that collagen stability is attained through hydroxyproline participating in hydrogen bonding (Brodsky and Ramshaw, 1997). A new peptide, 10x(proline-fluoroproline-glycine), containing 4(R)-fluoroproline in place of hydroxyproline is not hydrogen bonded to water molecules but is more thermostable than its natural counterpart proline-hydroxyproline-glycine (Holmgren et al., 1998).

2.4.1.1. Biosynthesis of collagen

The biosynthesis of fibril forming collagen starts with the intracellular expression of collagen genes in fibroblasts followed by RNA involved production of pro-collagen, the precursor form of collagen (McCormick, 1994). Within the rough endoplasmic reticulum, each α-chain, the primary unit of the pro-collagen fibril, twists in a left-handed helix (Baronas-Lowell, 2003) and three such chains are linked at the C terminals and wound together in a right- handed triple-helix to form a rod- like structure, 1.4 nm in diameter (Eyre, 1980; Bulleid et al., 1997; Smith and Rennie, 2007). Both C and N terminals of pro-collagen polypeptides have telopeptides, which are appendages with no helical arrangement and lack glycine at every third position.

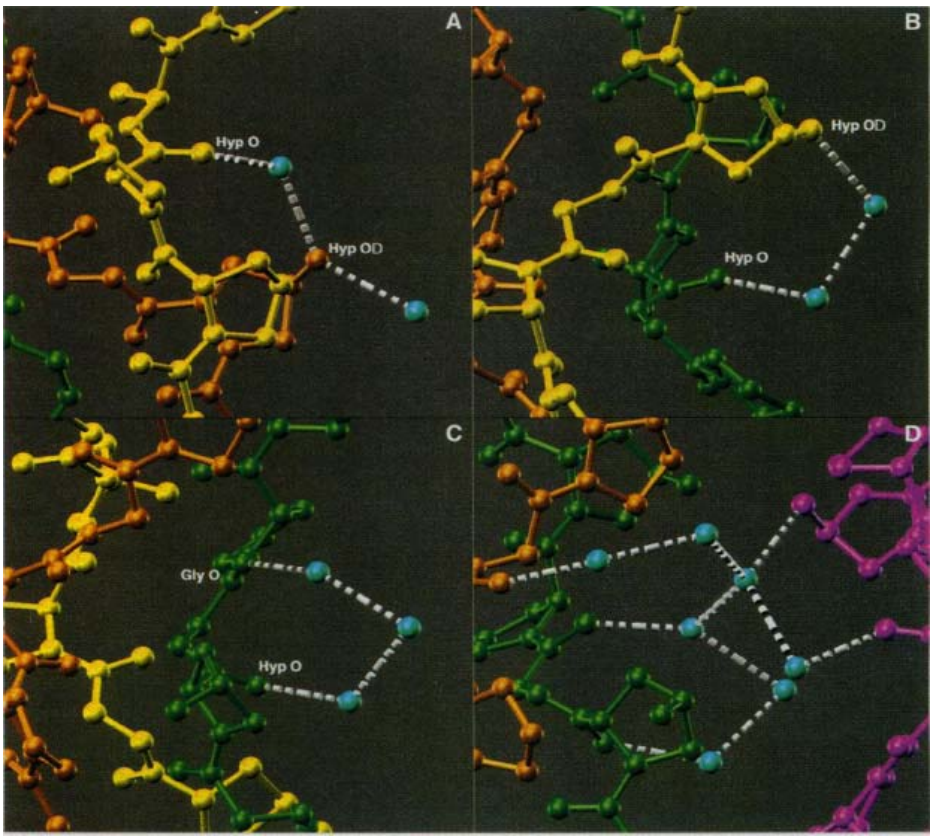


Figure 2.2 Water mediated H-bonding in collagen triple helix. (A) Two peptide chains are linked through one water molecule. (B) Two peptide chains are linked through two water molecules. (C) Two peptide chains are linked through three water molecules. (D) Intrachain linking through a network of water molecules. Figure 5 of Bella et al. (1994) reproduced with kind permission from the American Association for Advancement of Science (AAAS).

Telopeptides are also considered to be the primary sites of cross-linking in collagen molecules (Eyre, 1980; Baum and Brodsky, 1999). Before folding, collagen molecules undergo a series of posttranslational modifications starting from hydroxylation of a large number of proline and some lysine residues in the *Y* position of glycine-*X*-*Y* triplet followed by the glycosylation of certain hydroxylysines with galactose and glucosylgalactose. Also, asparagine residues on the C terminal of a polypeptide undergo glycosylation. Different types of collagen are hydroxylated or glycosylated to different degrees to determine the characteristics of each collagen type (Smith and Rennie, 2007).

Prolyl 4-hydroxylases are considered as the key enzymes that catalyse the formation of 4-hydroxyprolines. These enzymes are found in the lumen of the endoplasmic reticulum (Myllyharju, 2003). The hydroxylation of proline in the *Y* position produces *glycine-X-hydroxyproline* triplets in every five to six *glycine-X-Y* triplets of the α-chain. It is thought that having hydroxyproline at the *Y* position stabilizes the collagen triple helix through hydrogen bonding, in vivo (Nēmethy and Scheraga, 1986). Hydroxyproline is considered as the nucleation sites for combining α-chains with the correct dihedral angles (Berg and Prockop, 1973). Hydroxylation of lysine occurs through three different types of lysyl hydroxylases with no known specificity for collagen type (Wang et al., 2000). The degree and the site of lysine hydroxylation is tissue specific. For instance, lysine in both telopeptide and helical regions of collagen of cartilage and basement membranes are highly hydroxylated. In contrast, only lysines in telopeptide regions of bone collagen are highly hydroxylated (Avery and Bailey, 2008). It is also evident that proline and hydroxyproline undergo *cis*→ *trans* isomerization, which occurs through cis-trans prolyl isomerase (Bächinger, 1967; Kivirikko and Myllyharju, 1998; Baum and Brodsky, 1999).

2.4.1.2 Enzyme mediated cross-linking of collagen

After being released to the extra cellular space, the enzymes pro-collagen N-proteinase and pro-collagen C-proteinase truncate the N and C terminals of collagen molecules, respectively. Collagen thus formed, spontaneously self-assembles into cross-striated fibrils (Kadler, 1966; Canty and Kadler, 2005) (Figure 2.3). In this process, side chains of a few of the lysine and hydroxylysine molecules are converted to aldehydes by oxidative deamination through a lysiloxidase-catalyzed reaction (Robbins, 2007). Lysiloxidase is a copper dependant enzyme. It is believed that this enzyme specifically acts on lysine and hydroxylysine of *(Lysine) or*

Hydroxylysine-Glycine-Histidine-Arginine sequence located in opposite N- and C-terminals of quarter-staggered collagen molecules (Avery and Bailey, 2008).

Collagen is known to contain enzyme catalyzed and non-enzyme catalyzed cross-links (Gallop, 1972; Robbins, 2007). Tensile strength and functionality of collagen molecules are thought to be resulting from intermolecular cross-links (McCormick, 1999). The immature, bivalent, cross-links are formed through two major pathways, (a) allysine pathway, where lysine aldehydes are formed and (b) hydroxyallysine pathway, where hydroxylysine aldehydes are formed (Bailey and Peach, 1968). The allysine pathway is common in cross-linking of skin collagen (Eyre, 1984). Cross-linked products of allysine and hydroxyallysine pathways are called aldimines and ketoamines, respectively. Aldimines are effective intermolecular cross-links under physiological conditions but in vitro are easily cleaved by low pH and high temperature; this is a reason for the in vitro solubility of immature collagen. The most common lysine-aldehyde cross-link (aldimine), dehydro-hydroxylysino-norleucine, is formed through the reaction between lysil aldehyde and hydroxylysine of the telopeptide region of collagen (Bailey and Peach, 1968). Lysine aldehyde cross-links are formed in the N terminals of two adjacent peptide strands of collagen (intra molecular) and also between allysine and lysine or hydroxylysine in the helical regions of overlapping (inter molecular) collagen molecules (Kang and Gross, 1970). The hydroxyallysine (ketoamine) pathway is the most common cross-linking mechanism in skeletal muscles, myocardium and also in connective tissues that bear large mechanical loads (Eyre, 1984, McCormick, 1999). Ketoamines are stable at low pH and high temperature giving strength to the immature tissue (Bailey et al., 1998). Dehydro-histidino-hydroxymerodesmosine is a bivalent, aldimine cross-link commonly found in tendon and skin. It is an aldol condensation product between histidine and ε-amino group of hydroxylysine in the helical region of collagen. These immature cross-links are easily reduced in vitro, by sodium

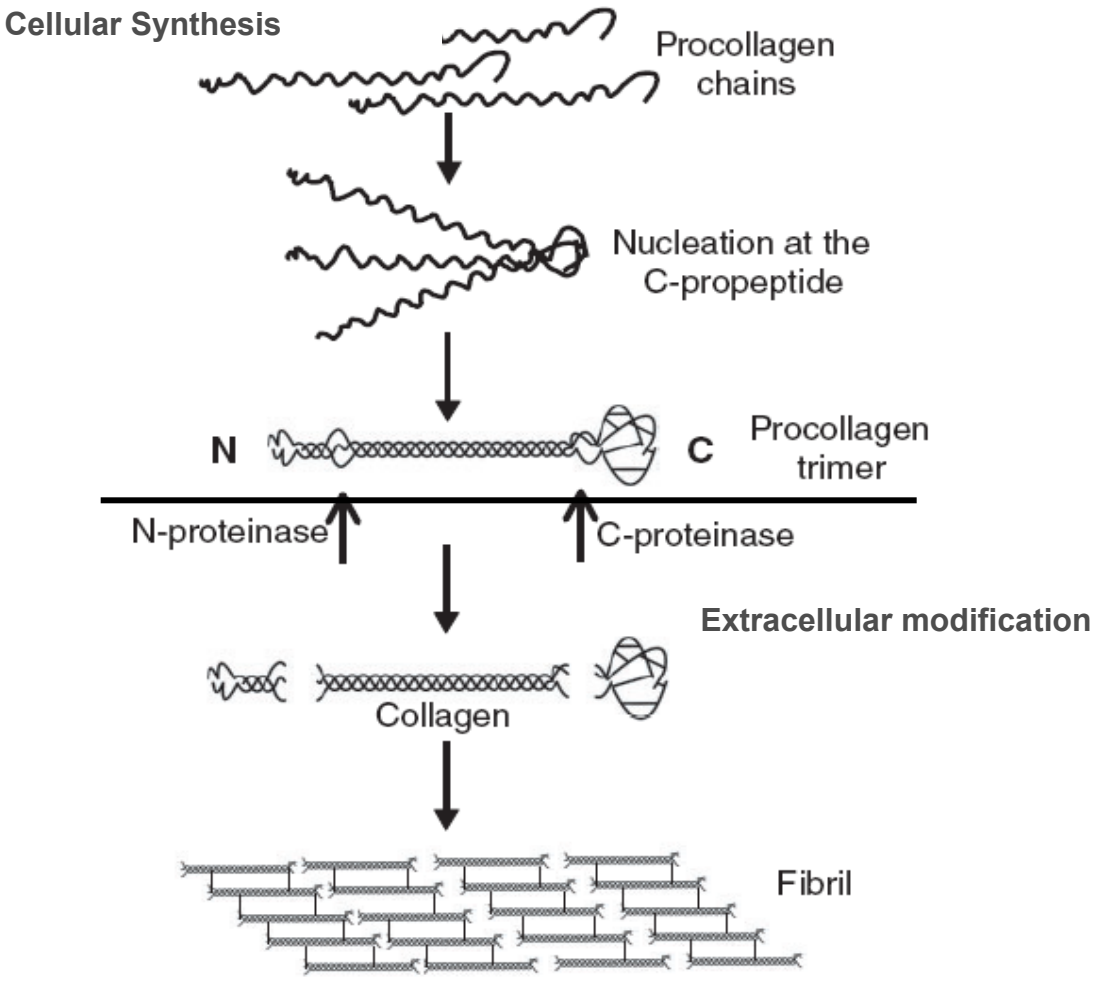


Figure 2.3 Process of collagen fibrillogenesis. Procollagen, the precursor of collagen is synthesized in the endoplasmic reticulum and assembled into trimers before transporting into extra cellular space. The initiation of fibrillogenesis is the truncation of N and C terminal peptides. Truncation may take place on the plasma membrane or extra cellular space. Fibrils are stabilized through cross-links. Figure 2 of Canty and Kadler (2005) adapted with kind permission from the Journal of Cell Science.

borohydride to form strong cross-links (Avery and Bailey, 2008). Another ketoamine cross-link, hydroxylysino-5-ketonorleucine, is formed between hydroxylysine aldehyde and the ε-amino group of hydroxylysine in the helical region of collagen (Bailey et al., 1998). However, lysil oxidase mediated cross-linking is a process that is active during the growth and maturation

of an animal, but not during the animal becoming old. After a critical age (in the life cycle), composition of collagen, degree of hydroxylation and nature of cross-links do not change (Freemont and Hoyland, 2007). This is demonstrated in collagen from old and osteoporotic bones. While bone collagen extractability increased with the age of humans (15-90 years), the concentration of enzymatic cross-links, dehydro-dihydroxylysinonorleucine and dehydro-hydroxylysinonorleucine, are reduced. However, the concentration of mature cross-links, pyridinolines do not change with the maturity of an animal (Oxlund and Ørtoft, 1996).

2.4.1.3 Mature cross-links of collagen

In addition to immature enzyme derived cross-links, presence of other mature and more stable forms of collagen cross-links is reported (Robins et al., 1973). Pyridinoline is a mature cross-link of collagen formed among three amino acid side chains originating from the carboxyl terminal and helical regions and also from amino terminal and helical regions of collagen molecules. In this process, lysine and hydroxylysine aldehydes are thought to combine with ε-amino group of another lysine to form a pyridinoline cross-link. When three side chains of lysines participate in the cross-link formation, it is called lysilpyridinoline (Fugimoto et al., 1977; Hanson and Eyre, 1996).

Pyrroles are ubiquitous, mature cross-links believed to be trivalent and are detected in the same regions where pyridinoline cross-links are present (Scott et al., 1981; Kuypers et al., 1992; Hanson and Eyre, 1996). Pyrroles are thought to form between the immature cross-link hydroxylysino-5-ketonorleucine and lysine-aldehyde in the telopeptide region of collagen. Hydroxylysino-pyridinoline is formed when hydroxylysino-5-ketonorleucine reacts with hydroxylysine aldehyde (Figure 2.4). Pyridinolines are formed when telopeptides are highly hydroxylated and pyrroles are formed when telopeptides are less hydroxylated (Bailey et al., 1998; Avery and Bailey, 2008). Histidinohydroxylysinonorleucine is another stable and non-

reducible cross-link of skin collagen formed through linking three amino groups from three different chains, Hyl-87 from α1(I), Lys-16 from α1(I) and most significantly His-92 from α2(I) (Mechanic et al., 1987). According to another investigation, the precursor for the histidinohydroxylysinonorleucine cross-link is the bivalent collagen cross-link, dehydrohydroxylysinonorleucine (Yamauchi et al., 1987). Ehrlich chromogen is another trivalent pyrrolic cross-link of collagen that is produced through the same pathway as pyridinoline cross-links are. The reaction between a bivalent keto-amine cross-link and lysine aldehyde in telopeptidal regions of collagen molecules leads to the Ehrlich chromogen (Scott et al., 1981; Kuypers et al., 1992).

2.4.1.4 Non-enzymatic cross-linking of collagen

Non-enzymatic glycation of collagen is a spontaneous reaction between sugars and lysine of collagen (Maillard reaction) to produce a range of cross-links and is illustrated in Figure 2.5 (Henle, 2005). These are also called advanced glycation end products (AGE) (Verzijl, 2000; Monnier et al., 2005). AGE products are polycyclic, fluorescent compounds (Hodge, 1953) and in vivo they are processed over weeks. Therefore, AGE products are (adversely) formed with long-lived extra cellular matrix proteins such as collagen (Vitek et al., 1994; Singh et al., 2001) and the highest AGE levels are observed in tissues with slow turnover, such as tendon, skin, bone, amyloid plaques and cartilage (DeGroot, 2004). AGE cross-links are limited to helical regions of collagen and do not involve terminal, telopeptide areas (Robbins, 2007). Also, oxidation of proteins due to ageing and pathology (diabetes) is considered as a reason for AGE production (Dyer, 1993). Verzer (1964) first reported the age related AGE cross-linking in tendon collagen. Pentosidines, N^{ε}-(carboxymethyl)lysine, and N^{ε}-(carboxyethyl)lysine, are common AGE products of ageing cartilage. They are formed when one lysine and one arginine moiety cross-linked by a pentose (Verzijl et al., 2000). Other AGE cross-links such as glyoxal-

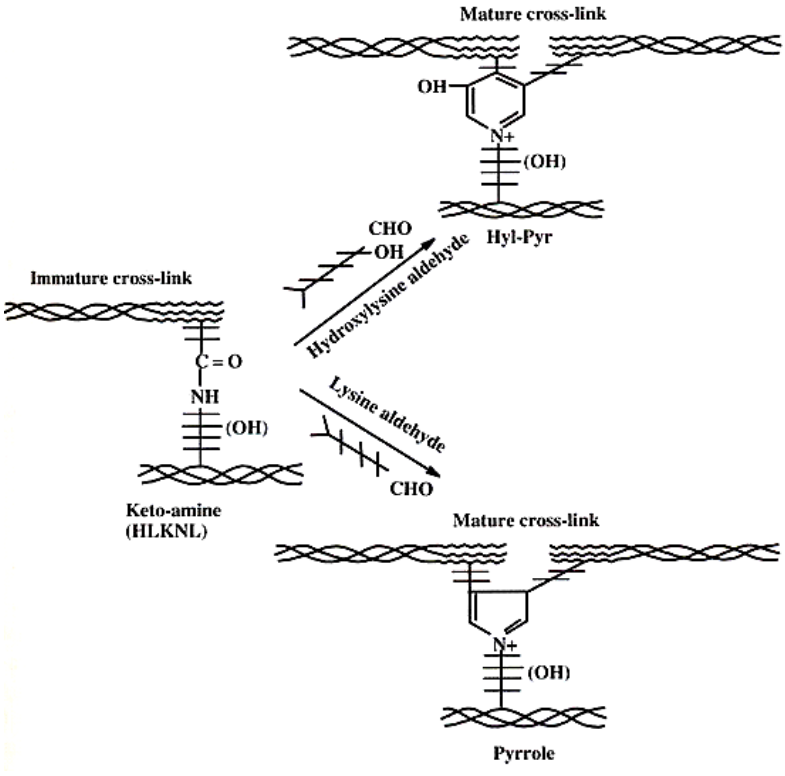


Figure 2.4 Chemical reactions to form trivalent collagen cross-links, pyrrole and hydroxylysino-pyridinoline (Hyl-Pyr). Divalent ketoamine cross-link, hydroxylysino-keto-norleucine (HLKNL), combines with either telopeptide hydroxylysine aldehyde to form hydroxylysino-pyridinoline or with lysine aldehyde to form pyrrole. Figure 4.2 (page 85) of Avery and Bailey (2008) reproduced with kind permission from Springer Science and Business Media.

lysine dimmer and methylglyoxal-lysine dimmer are detected in lens capsule collagen (Frye et al., 1998).

Proteoglycans in cartilage are also glycated to form pentosidines (Pokharna and Pottenger, 1997). The results of glycation are reduced collagen solubility and increased matrix stiffness (Reddy, 2004 a, Reddy, 2004 b; Valcourt et al., 2007). As recently elucidated, AGE cross-linking in humans may be induced by the diet rich in AGE products through post-translational modification of proteins (Henle, 2005) even though, receptors on macrophages are reported to bind AGE products (Vlassara et al., 1985).

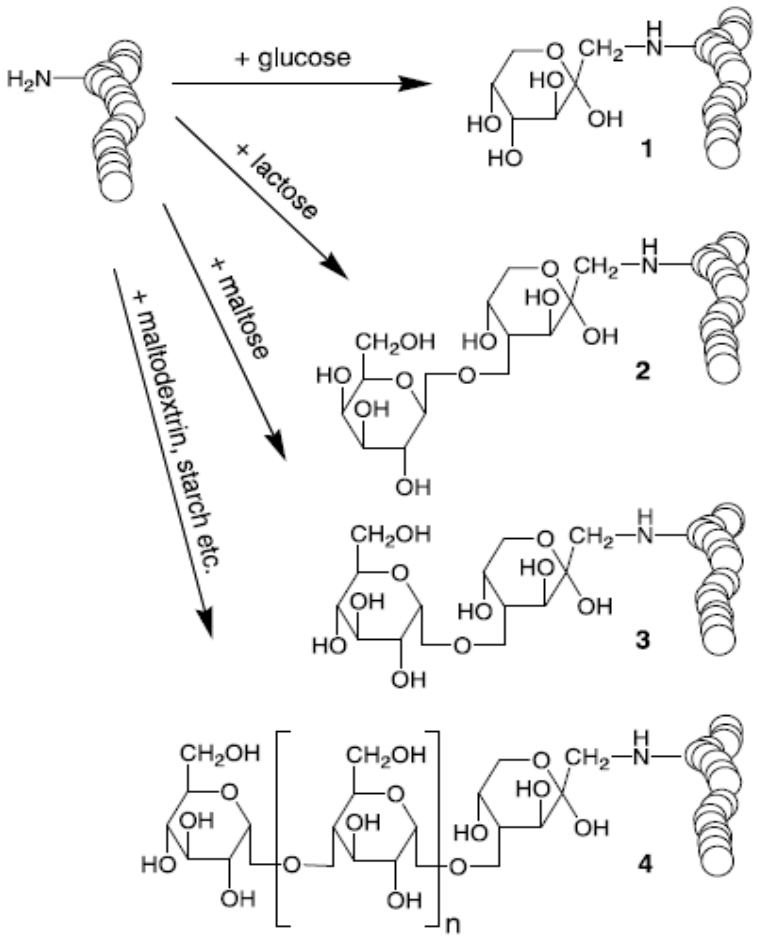


Figure 2.5 The reaction of protein bound lysine with sugars. Lysine reacts with (1) glucose to form N-ε-fructosyllysine, (2) lactose to form N-ε-lactulosyllysine, (3) maltose to form N-ε-maltulosyllysine and (4) maltose or starch to form protein-linked olygosaccharides. Figure 1 of Henle (2005) reproduced with kind permission from Springer Science and Business Media.

2.4.1.5 Collagen types

Despite the similarities in molecular structure, collagen is a complex protein with about 28 different types already identified in different tissues (Veit et al., 2006). As mentioned, a molecule of collagen consists of three strands of peptides and each strand is also designated as

an α chain. These strands unite in different forms, for example, (a) combine three apparently identical chains such as $[\alpha1(I)]_3$, $[\alpha1(II)]_3$, $[\alpha1(III)]_3$, (b) combine three different chains [(α1) (α2) (α3)] and most commonly (c) combine two identical chains with one different $[\alpha1(I)]_2$ α2]. The nomenclature of collagen chains is such that α1 chains in different types of collagen (type I, II or III.) are labelled as α1(I) or α1(II) or α1(III) because α chain differs among collagen types (Gallop et al., 1972). Lysine and hydroxylysine residues in a given position of α1 and α2 chains do not always form aldehydes and thereby, having and not having aldehydes on a particular location of α1 and α2 chains determines the heterogeneity of collagen (Piez et al., 1966). The naming of vertebrate collagen as type I, II, III (and so on) indicates the order in which they were discovered (Boot-Handford and Tuckwell, 2003).

Collagen types show tissue specific distribution pattern, for example, types I, III, V and VI are detected in both endomysium and perimysium during the development of bovine intramuscular connective tissue. Collagen type IV is located exclusively in the endomysium, not in perimysium (Nishimura et al., 1997). Through immuno-histochemical staining, Nakamura et al. (2007) has confirmed that perimysium from mature animals mostly contain collagen type III and I. Collagen type IV is detected in endomysium and type V and VI are detected in both perimysium and endomysium. Epimysium from both mature and pre-natal tissues is known to contain collagen types III and I (Shellswell et al., 1980; Light and Champion, 1984).

Collagens are also classified on the nature of their aggregated form. The 'fibrillar collagen' is a long (300 nm) rod-like molecule that self assembles to form parallel, quarter-staggered structures with a characteristic banding pattern every 67 nm and is observed in collagen types I, II, III, V and XI. The 'net-work collagen' is 400 nm long and forms a chicken wire network as observed in collagen types IV, VIII and X. The 'filamentous collagen' consists of loosely packed filaments having a 100 nm axial repeat as observed in collagen type VI. The 'fibril

associated collagen' does not form fibres but is linked to other fibre forming collagens (Bailey et al., 1998). The most common form of fibrillar collagens consists of a triple helical domain edge by two terminal globular domains known as N- and C-telopeptides that do not exhibit *glycine-X-Y* repeat (Wess, 2005; Boot-Handford and Tuckwell, 2003). Fibrils of collagen are observed in complex three-dimensional arrays, as parallel bundles in tendons and ligaments, as orthogonal lattices in cornea and as concentric weaves in bones (Canty and Kadler, 2005).

It was thought that during evolution, the observed molecular architecture of collagen developed and remained within many species of animals. Thus, it provides the optimum resistance to shear deformation (Buehler, 2008). The triple helix is regarded as the basic motif of collagen with the ability to adapt to functional needs of tissues (Brodsky and Persikov, 2005). This is well explained by examining molecular organization of collagen in bones (Rho et al., 1998) (Figure 2.6). The quarter-staggered arrangement of fibril forming collagen molecules, which has a 27 nm overlap area and a 40 nm gap between the ends of 2 molecules to provide the basis of the 67 nm banding pattern is common to type I collagen. The adaptive advantage of collagen is such that the 40 nm spacing in bone collagen is mineralized.

2.4.1.6 Collagen metabolism

Collagen in vivo is responsible for several functions; (a) provides tensile strength to tissues through physical and chemical interactions within a molecule and with other extra cellular matrix components, (b) provides tissues with flexibility through a unique molecular organization that facilitates sliding of the molecules past one another, (c) entraps proteoglycans and body fluids to prevent their uncontrolled movement as in cartilage, (d) facilitates platelet aggregation, blood clotting and prevent mobilization of blood clots, (e) acts as a substrate for the mineralization of bones and (f) facilitates cell differentiation during foetal vertebral development (Minor, 1980).

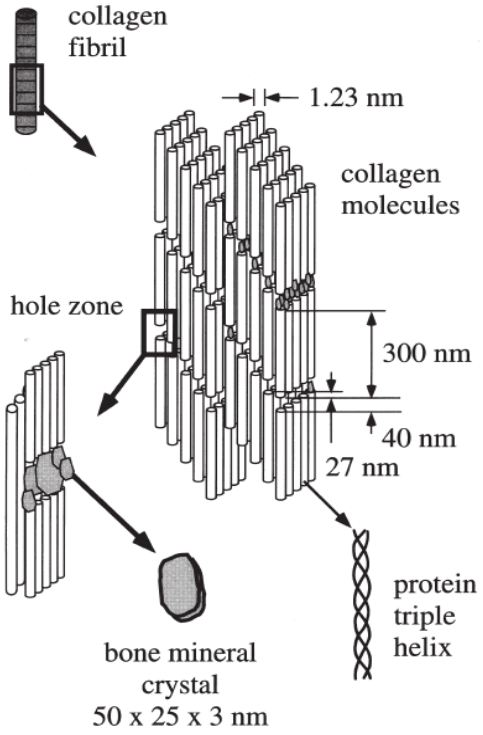


Figure 2.6 Arrangement of collagen type I molecules in bone. The universal periodicity of 67 nm of fibril forming collgen is a result of 40 nm spacing between 2 ends of molecules and the 27 nm overlap region. The adaptive advantage of this arrangemnt in bone is apparent through the mineralization of 40 nm gaps. Figure 2 of Rho et al. (1998) reproduced with kind permission from Elsevier Limited.

Collagen is continuously remodelled in vivo during the growth and development of an animal and depolymerization occurs both extra cellularly and intra cellularly. Extra cellular degradation happens through a family of enzymes referred to as matrix metalloproteinases (Woessner, 1991). Intracellular degradation of newly synthesized collagen, before it is secreted out of the cell, occurs in lysosomes through lysosomal enzymes (Berg et al., 1984). Collagenases in tissue are hypothesized to exist in three different forms; (a) as precursors of enzymes later activated by other proteolytic enzymes or by the removal of inhibitors bound to them, (b) as active free collagenases in tissues and (c) as active collagenases bound to the substrate, which cleave collagen under optimal conditions for enzyme activity (Pérez-Tamayo, 1978). Collagenases of animal origin split collagen molecules between amino acid residues 772

and 773 to produce one long and one short peptide and these are susceptible to other proteolytic enzymes (Pérez-Tamayo, 1978).

The rate of collagen turnover varies with the tissue. For example, in periodontal ligament about 10% daily turn over (calculated as a ratio between radioactivity of collagen degraded and that of precursor pool of amino acids) occurs and intra cellular degradation of collagen occurs minutes after synthesis to allow adaptive advantages. Thereby, collagen degradation regulates the collagen mass in a tissue (Laurent, 1987). About 55% of the newly synthesized collagen in rat skin disappears from the soluble collagen pool due to maturation to form insoluble collagen and the other 45% is lost due to degradation (Molner et al., 1986). Collagen synthesis in skeletal muscles is induced by acute exercises indicating an adaptive response. Simultaneously, lysyl oxidase expression also is increased indicating cross-linking of collagen (Heinemeier et al, 2007). Also, maturity of an animal is known to influence collagen metabolism, for instance, synthesis of collagen type I is impaired in mature human skin and that changes the ratio of collagen type I:III and also decreased the number of collagen fibre bundles (Lovell et al., 1987). The other changes observed with ageing include an increase in the area of arterial collagen fibre bundles with a concurrent reduction in the number of collagen bundles, merging of collagen fibres and reduced branching in bundle network (Gudiene et al., 2007). Animal nutrition is reported to have no impact on mature collagen content in skeletal muscles (Smith and Rennie, 2007).

2.4.2 Proteoglycans

Proteoglycans are a heterogeneous mix of protein and carbohydrate moieties with the common feature of a protein core and at least one chain of glycosaminoglycan attached to it. Proteoglycans are generally extra cellular matrix components and participate in cell adhesion. The significance of proteoglycan to this discussion is that collagen (as a protein) participates in

proteoglycan formation during the procollagen stage or later when fibrils are formed. The interaction between collagen and glycosaminoglycan, most likely electrostatic in nature, accelerates the collagen fibril formation (Scott, 1988). Proteoglycans of non-collagen origin also bind to collagen, for example, decorin binds to collagen type VI (Bidanset et al., 1992). Each glycosaminoglycan is a polymer of disaccharides i.e. D-acetylglucosamine, uronic acid, D-xylose, D-mannose, L-fucose and D-galactose (Lindahl and Höök, 1978). Chemical structures of some of the disaccharides that participate in glycan formation (chondroitin sulphate, dermatan sulphate and keratan sulphate) are shown in Figure 2.7 (Scott, 1988).

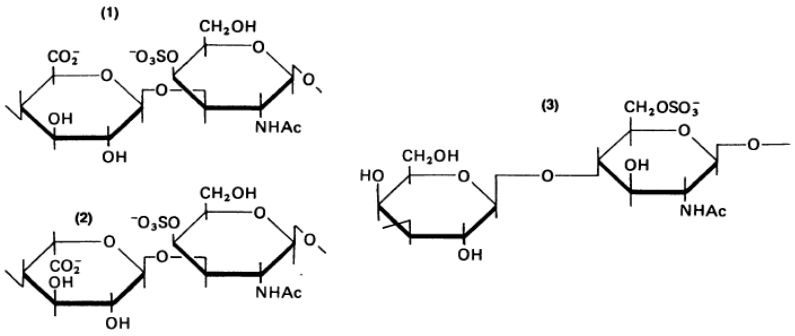


Figure 2.7 Glycans of proteoglycans consist of repeating units of disaccharides. (1) Chondroitin sulphate, (2) Dermatan sulphate and (3) Keratan sulphate. Figure 2 of Scott (1988) reproduced with kind permission from Portland Press Ltd.

Proteoglycans are classified according to their structure and function; (a) serglycine has a small core protein and about 14 glycosamonoglycan chains attached to it, (b) heparan sulphate has a separate domain to bind to the cell membrane (c) decorin intervenes in binding collagen and fibronectin and (d) hyaluronic acid moieties are generally large. Glycosaminoglycans, sugar moieties, are of four different types, heparin (forms heparan sulphate), chondroitin sulphate (forms dermatan sulphate), keratan sulphate and hyaluronic acid (Ruoslahti, 1988).

Composition of proteoglycans varies at different stages of skeletal muscle development. Chondroitin sulphate in the proteoglycan versican is common during the early phase of muscle development and also during regeneration of muscles. Synthesis of small dermatan sulphate molecules in the proteoglycan decorin begins at the latter stages of muscle development and then decorin binds to collagen. Heparan sulphate also is located in developing muscles and plays a role in myoblast proliferation (Carrino, 1998).

Major proteoglycans of endomysium and perimysium of prenatal and postnatal bovine skeletal muscle are heparan sulphate and dermatan sulphate, respectively (Nishimura et al., 1997). Galactosaminoglycans with dermatan sulphate is the major type of proteoglycans detected in porcine skeletal muscle epimysium (Nakano et al., 1996). It is thought that the proteoglycan decorin controls the fibrillogenesis of collagen type III and I (Nishimura et al., 2003). On the other hand dermatan and keratan sulphate are observed in close association with Type I, II and III fibrillar collagen where they are electrostatically bound to the gap between quarter-staggered collagen molecules, but not incorporated into collagen fibrils. Each proteoglycan is known to bind to a specific site on collagen (Vogel et al., 1994; Scott, 1988, Thieszen and Rosenquist, 1994).

Glycosaminoglycans have a large 'hydrodynamic volume', that is the ability to bind large volumes of water and swell relative to their weight. The electronegative charges on the sulphated glycosaminoglycans repel one another and attract water (Wirth and Rudert, 1996). Proteoglycans retain more water when collagen in cartilage is degenerated or is damaged due to pathology or external load (Inerot et al., 1978; Torzilli et al., 1999). Between glycosaminoglycans of similar molecular weight, dematan binds more strongly to collagen than chondroitin sulphate and the affinity of these molecules to collagen increases as molecular weight increases (Öbrink and Sundelöf, 1973). Garg et al. (1989) has reported that the presence of high molecular weight proteoglycan during collagen fibrillogenesis increases the tensile strength of fibres more than the low molecular weight chondroitin sulphate proteoglycan.

Age of the animal brings changes to proteoglycans in tissues; in articular cartilage the number and size of chondroitin sulphate chains decreases and the number and size of keratan sulphate increases with advancing age (Hardingham and Bayliss, 1990). Similar increase in keratan sulfate and reduction in chondroitin sulfate is common to ageing spine (Adams et al., 1977). In chondrocytes of ageing articular cartilage, synthesis of proteoglycan reduces (DeGroot et al., 1999) as a result of chondrocyte senescence (Martin and Buckwalter, 2002).

2.4.3 Elastin

Elastin provides resilience and the elastic recoiling properties to tissues that are subjected to repetitive expansion and physical stress (Rodgers and Weiss, 2005). Elastin normally exists in a contracted state but is capable of extension to twice that of the contracted state (Pearson et al., 1989). The basic molecular unit of elastin is a polypeptide with a molecular weight of about 72 kDa and is referred to as tropoelastin. Without further cleavage, these polypeptides are incorporated to form elastin fibres (Bressan and Prockop, 1977). As in collagen, glycine provides ⅓ of amino acids of elastin but is not evenly distributed along the peptide chain.

Hydroxyproline content of elastin is less than that of collagen (Uitto, 1979) and about 33% of elastin is glycine, 10-13% proline and other amino acids with hydrophobic side chains account for over 40% (Pearson et al., 1989). Elastin is a highly insoluble protein rich in both hydrophobic and hydrophilic amino acids. The hydrophobic sequence is responsible for the elasticity of elastin when water molecules absorb on to hydrophobic amino acids and the hydrophilic alanine rich portion forms α helices and participates in cross-linking (Gosline, 1978; Eyre, 1984). Collagen and elastin are similar in their ability to form cross-links and lysil oxidase is the essential enzyme for the formation and repair of elastin, which oxidizes lysine to initiate covalent cross-linking (Kagan et al., 2003). Lysil oxidase converts lysine to allysine and two such allysine molecules combined to form an allysine aldol. Further condensation and subsequent oxidation produce desmosine and isodesmosine (Eyre, 1984).

The elastin content in muscles is very small and generally is insignificant compared to the percentage of collagen (Mitchell et al., 1928). During prenatal and postnatal growth of rat skeletal muscle elastin is observed in epimysium, perimysium and endomysium, but amounts do not vary with growth (Kurose et al., 2006). Perimysial and epimysial elastin has two structural forms: (a) 1-10 μm diameter, coarse fibres aligned with the long axis of the muscle fibre and (b) 1-2 μm diameter fine elastin fibres aligned at an angle to the long axis of muscle fibres (Rowe, 1986). Elastin is produced only up to early maturity and any variations during the rest of the lifetime are due to degradation or degeneration, typical of ageing (Oleggini et al., 2007). Elastic fibres are progressively hydrolysed during maturation, ageing and pathology. To that effect, several elastases are detected in increasing quantities with ageing in arterial smooth muscle cells and skin fibroblasts (Robert et al., 1984). It is proposed that the upper limit for elastic properties of elastin is limited to 100-120 years (Robert et al., 2008).

2.4.4 Fibrillin

Fibrillin is a glycoprotein (~350 kDa) stabilized through intra chain disulfide bonds. It is linked to glucosamine and provides the backbone structure of micro fibrils. Fibrillin is a major structural component of extra cellular matrix and produces 10-12 nm long elastin fibrils (Jordan et al., 2006). Fibrillin is widely distributed in connective tissue of skin, lung, kidney, vasculature, cartilage, tendon, muscle and cornea and show a periodicity similar to that of interstitial collagen (Sakai et al., 1986). Fibrillin is known to participate in cell adhesion through cell-specific receptors (Bax et al., 2003).

2.4.5 Fibronectin

Fibronectin is a structural glycoprotein present in the extra cellular matrix and plasma and is synthesized by fibroblasts (Gatchalian et al., 1989). This protein plays a central role in cell adhesion (Ruoslahti, 1988) and the ability of fibronectin to bind cells is attributed to a tetra peptide sequence, *L-arginyl-glycyl-L-aspartyl-L-serine* (Pierschbacher and Ruoslahti, 1984). Fibronectin also binds to collagen, glycosaminoglycans and proteoglycans (Stathakis and Mosesson, 1977; Engvall and Ruoslathi, 1977; Perkins et al, 1979). In vitro, fibronectin is found to have a high affinity to collagen types I and III. It is also proposed that fibronectin on cell surfaces act as cell adhesion sites to extra cellular matrix collagen (Engvall et al., 1978).

2.5 Thermal properties of muscle proteins

2.5.1 Thermal properties of myofibrillar proteins

Since cooking is the major means of processing meat for consumption, it is pertinent to discuss the thermal properties of major meat proteins, myosin and actin. During heating (between 40-90 ºC) in an aqueous media (pH 5.5), isolated myofibrils do not decrease in length but their fibre diameter is decreased. Also, the volume of muscle fibres is decreased by ½ and water content is decreased by 60% (Bendall and Restall, 1983). Heat induced unfolding of chymotrypsin-

digested myosin has produced three protein transition peaks; the main transition peak is at 52.5 ºC and is thought to be resulting from the melting of myosin S1 and S2 regions. The two trivial transition peaks are observed at 46 and 57 ºC and are considered as a result of melting of tail region of myosin (Samejima et al., 1983). After excluding sarcoplasmic and connective tissue proteins, the rest of the muscle proteins have produced three (pH dependant) endothermic peaks. The peaks I (57.8 ºC) and II (63.9 ºC) are attributed to myosin denaturation and the peak III (74.2 ºC) is attributed to actin denaturation (Wagner and Aňon, 1985). Myofibrillar proteins are reported to denature after exposure to freezing temperatures (-20 ºC) and to high-pressure (200 Mpa) as observed in thermograms from differential scanning calorimetry and transmission electron micrographs but after similar treatments connective tissue proteins were not denatured (Fernández-Martín et al., 2000).

2.5.2 Thermal properties of collagen

Collagen is the most abundant connective tissue protein and undergoes a phase transition from an organized crystalline state to an amorphous state with random coils during heating (Garret and Flory, 1956; Flory and Garret, 1958). Collagen thermal denaturation is reported to take place within a range of temperatures; for example, denaturation of endomysial collagen begins at 50 ºC and is completed at 70 ºC but the denaturation of perimysial collagen begins at 70 ºC (Schmidt and Parrish, 1971). According to scanning electron micrographs, denaturation of perimysial collagen takes place at 70- 80 ºC (Cheng and Parrish, 1976; Leander et al., 1980) and shrinkage of collagen rich muscle strips begins at 64 ºC which intensifies at 94 ºC (Bendall and Restall, 1983). Using a combined NMR and DSC study, Rochdi et al. (1999) have demonstrated that thermal changes do not take place in bovine epimysial collagen up to 50 ºC. At 60 ºC, collagen thermal denaturation is possible only when moisture content is >1.5% of the dry weight of collagen. Similarly, at 70 ºC, the amount of collagen that undergoes phase transition is a function of water content (Rochdi et al., 1999). As such, thermal denaturation temperature of

collagen is a complex phenomenon influenced by both pH and moisture content of the medium. Referring to peak thermal denaturation temperature (*Td*), Wright and Humphrey (2002) stated that "Unfortunately, citing these temperatures may lead some to conclude that there is a specific thermodynamically defined temperature for the denaturation of collagen. Thus, although often recorded, *Td*, is neither a property of the material nor it is uniquely defined for a given sample".

Thermal stability of collagen from rat-tail tendon is reported to be influenced by glycosylation, because time taken to break a glycosylated collagen fibre increases with the age of the animal and the strength of glucose treatment (Yue et al., 1983). The ability of glucose mediated cross-linking to increase the stability of collagen is verified by the observation that insulin treatment decrease the thermal stability of collagen from diabetic rats (Andreassen et al., 1981). Collagen cross-links, pyridinoline and Ehrlich chromogen, also were reported to correlate well with thermal transition temperature and hydrothermal isometric tension of calf tendons (Horgan et al., 1990). Using dehydrated samples of bovine Achilles tendon, Okamoto and Saeki (1964) reports that tendon collagen has three different levels of fibre organization and which undergo phase transition at different temperatures, (a) less organized unsteady "crystalline region" with a broad range of melting temperatures between 80-180 ºC, (b) "an amorphous region with a second order phase transition point" at 120 ºC and (c) "stable crystalline region" having a clear melting temperature close to 200 ºC and this region is thought to be stabilized through ionic bonds and dense cross-linking.

2.6 Measurements of protein denaturation

2.6.1 Differential Scanning Calorimentry (DSC)

DSC is a popular method to determine thermal denaturation of bio-molecules. According to Höhne et al. (2003), 'DSC means the measurement of the change of difference in the heat flow rate to the sample and to a reference sample while they are subjected to a controlled temperature

programme'. Contemporary calorimeters are equipped with twin cells, where one holds the sample and the other holds the reference material. This system is heated at a constant rate, adiabatically. Energy is absorbed into the system when heat capacity of sample cell and reference cell differs from each other in order to zero the temperature difference between cells. This difference in energy is reported as the heat capacity (Js^{-1}) of the sample (Jelesarvo and Bosshard, 1999).

DSC is used to quantify and follow the structural transitions of both bio-and non-bio polymers, chemicals and other materials due to several reasons; its ability to be used at a wide range of temperatures, for example, from –180 ºC to 725 ºC, non-destructive sample preparation methods, and shorter analysis time. The major limitation of DSC is that it measures only the total or average heat flow rate of overlapping transition processes and such that separation of individual processes is unlikely (Thomas, 2005). Despite that, DSC is used to investigate transitions in lipids in lipid mixtures (Mabrey & Sturtevant, 1976), gelatinisation properties of starches of different origins (Biliaderis et al., 1980) and thermal stability of meat proteins (Quinn et al., 1980) and plant proteins (Harwalkar and Ma, 1987). Modulated DSC (MDSC) has the advantage of simultaneously measuring the total heat flow as well as the heat capacity and other kinetic changes such as glass transition (Van Assche et al., 1995).

2.6.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy is a popular technique to study the conformational changes in protein structure. Infrared (IR) spectrum of proteins and peptides are linked to the peptide bond and thus referred to as "amide bands". Spectral bands designated as amide A, amide B and also bands listed from I to VII are attributed to in-plane stretching of C=O, C-N, N-H bonds, bending of O-C-N, C-N-H, C=O and N-H bonds and also out-of-plane torsion in C-N bonds (Arrondo et al., 1993). Amide I band is regarded as responsive to the

secondary structure of a protein (Miller et al., 2003). In general, reduced peak intensity of IR spectra is regarded as an indication of some degree of protein denaturation (Renugopalakrishnan et al., 1989, Friess and Lee, 1996).

The amide I region of the collagen (type I) triple helix does not represent the typical amide I band of α-helix or β-turn of other proteins but can be fragmented to three other bands with peaks at 1660, 1643 and 1633 cm^{-1} (Payne and Veis, 1988). According to Pelpis et al. (1996), the amide I band of collagen (1600-1660 cm^{-1}) is resulting from the vibration of C=O bond and amide II (~1550 cm^{-1}) and Amide III bands (1320-1220 cm^{-1}) are resulting from N-H bending and C-N stretching, respectively. In the presence of hydrogen bonds, amide 'A' band of collagen (3323 cm^{-1}) is shifted to a lower frequency (~3233 cm^{-1}) (Figure 2.8). However, according to Potter et al. (2001) collagen type II shows IR spectra typical to other proteins, having amide I band at 1649 cm^{-1}, amide II band at 1545 cm^{-1} and amide III band at 1339 cm^{-1}. The contribution of collagen cross-links to the IR spectra was investigated (Paschalis et al., 2001); after resolving the amide I region for its components the sub-bands at 1660 and 1690 cm^{-1} were attributed to pyridinoline and dehydrodihydroxylysinonorleucine cross-links. FTIR was used to study the effect of heating on conformational changes of intact cartilage collagen (Youn and Milner, 2008).

Accordingly, the intensity of absorption peaks of amide I and II decreases as proteins are denatured. Also, concurrent exposure to high temperature and pressure decrease the β-sheet and α-helical contents of β-lactoglobulin but increase the disordered arrangements (Panick et al., 1999). The far-IR spectra, 400-300 cm^{-1}, of denatured collagen samples cast at 4, 23 and 60 ºC

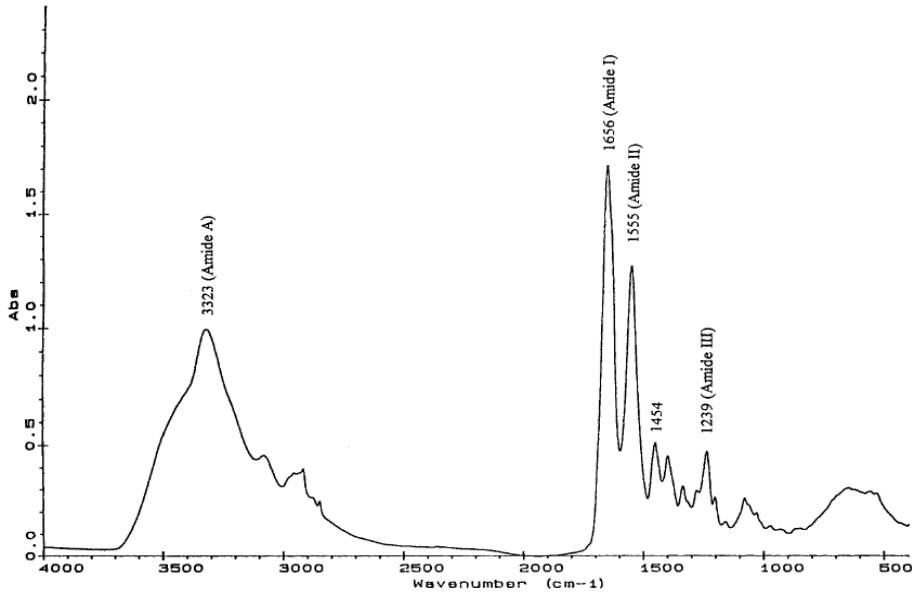


Figure 2.8 FTIR spectra of acetic acid soluble bovine serosa collagen. The spectra, amide I, II and III reveals the conformation of polypeptide; Amide I (1600-1660 cm^{-1})- vibration of carbonyl group, Amide II (~1550 cm^{-1})- N-H bending and C-N stretching and Amide III (1320-1220 cm^{-1}) –N-H bending and C-N stretching. Amide A band- (3323 cm^{-1}) shows the N-H stretching in the absence of hydrogen bonds. Figure 2 of Plepis et al. (1996) reproduced with kind permission from John Wiley & Sons.

had demonstrated the declining stability of helical structures of collagen as temperature was increased, where flattening of the peak (at 345 cm^{-1}) was observed for samples cast at 60 ºC (Sanui et al., 1974).

Instead of investigating purified proteins, emphasis of FTIR application were later drawn to direct investigation of biological material and body fluids, for example, cartilage, matter from the central nervous system, lymphocytes, saliva and urine. IR spectra of tissues are used in pathological investigations and chemical quantification (Jackson et al., 1995; Jackson et al., 1997; McIntosh et al., 1999; Potter et al., 2001; Camacho et al., 2001). However, as Jackson et al. (1995) states, "…. as the amide I absorptions of all proteins in tissues are observed

simultaneously it is usually not possible to assign particular amide I absorptions to particular secondary structures within one protein in IR spectra of tissues". According to the available literature, IR spectra of muscle connective tissues are seldom studied. However, an insight to the IR spectra of connective tissue of intact and processed bovine muscles is available (Bertram et al., 2006; Wu et al, 2006; Böcker et al., 2007; Kohler et al., 2007; Wu et al., 2007). After distinguishing spectral areas of perimysium and muscle fibres on intact tissue, Kohler et al. (2007) have reported that amide I band of perimysium is around 1695 and 1677 cm^{-1}, amide II bands is around 1512 and 1528 cm^{-1} and amide III bands is around 1204 and 1283 cm^{-1}. Amide I region of muscle proteins (1700-1600 cm^{-1}) is reported to be very sensitive to thermal treatments and the intensity of amide bands I and II (1652 and 1660 cm^{-1}) is decreased with the increasing temperature (Bertram et al., 2006). After rapid heating of porcine meat, spectral intensity of aggregated β-sheets and random structures are increased while decreasing the spectral intensity of α-helical content (Wu et al., 2007). Processing conditions such as post-mortem ageing and salting of meat have influenced the spectral changes by increasing α-helical and β-sheet content, respectively (Wu et al., 2006).

2.7 Tenderness/toughness of meat

Tenderness is a quality attribute of meat that is of paramount importance to consumer satisfaction. Tenderness or toughness of meat has been a topic of discussion for over a century. Lehmann (1907) was reported to carry out the first study on meat tenderness. Generally, toughness of beef is attributed to both muscle fibres and connective tissues (Marsh, 1977; Rowe, 1981; McCormick, 1994; Tornberg, 1996; Christensen et al., 2000) and these two areas are discussed below.

2.7.1 Toughness of muscle fibres

Contribution of muscle fibres to toughness of meat has been investigated for many years (Brady, 1937; Smulders et al., 1990; Torrescano, 2003). The relationship between muscle fibre

characteristics and the tenderness had been studied as early as 1930s and significant correlations were reported between the sizes of the muscle fibre bundles and shear stress, texture score and tenderness score (Brady, 1937). Beef toughness is observed to vary among muscles from the same animal (Torrescano, 2003). In muscles undergoing rigor mortis, the major contractile proteins actin and myosin are bonded and believed to be contributing to toughness. Sarcomere length is directly governed by the actomyosin bonding and the amount of tension generated in muscles at rigour. The impact of actomyosin bonding is clearly demonstrated in cold shortening. According to Smulders et al. (1990), beef toughness is increased with the severity of cold shortening of muscle fibres and increased tenderness is positively correlated with the sarcomere length. Compared to conventional carcass cooling, "very fast chilling' of beef in 3.4% NaCl solution at -2 ºC results in cold shortening of meat and high shear force values (Moeseke, et al., 2001). Ultimate pH of the meat is a good indicator of toughness/tenderness; the higher the pH (pH 5.4-6.8 range) the lower the shear force is and also the higher the sensory tenderness is (Silva et al., 1999).

Holding meat at low temperatures during the first few weeks post-mortem increases tenderness due to weakened myofibrillar proteins (Geesink et al., 1995; Nagaraj et al., 2005). The enzyme-mediated ageing and resulting tenderization of meat is widely discussed. Among enzymes, the calpain family of enzymes are reported to play a major role in the regulation of post-mortem proteolysis and tenderness (Hopkins and Thompson, 2002). Also, the inactivation of this protease is reported to increase the toughness (Hopkins and Thompson, 2001). From the family of calpains, μ calpains are responsible for the most of the post-mortem proteolysis (Geesink et al., 2006). However, according to other reports both m (144 h) and μ calpains (48 h) become inactive during early post-mortem when pH is above 5.8 and sarcoplasmic Ca^{2+} concentration is low (Kanawa et al., 2002, Camou et al., 2008). Cathepsin B and D are responsible for myosin degradation (Schwartz and Bird, 1977). It is also proposed that other proteases, for example,

chymotrypsin-like and trypsin-like enzymes play a gradually increasing role up to 7th day post-mortem (Lamare et al., 2002). Among the proteins degraded, myosin heavy chains are hydrolysed to produce 125 and 145 kDa peptides and the proteolysis is completed by the 14th day post-mortem (Bechtel and Parrish, 1983). Post-mortem degradation of nebulin and titin are completed by the 7th and 14th day, respectively (Huff-Lonergan et al., 1995). Post-mortem degradation of troponin-T is faster in stretched muscles with long sarcomeres than in shortened muscles and the process is completed by the 10th day post-mortem (Weaver et al., 2008).

Myofibrillar fragmentation is observed during ageing of meat and is completed within the first few days post-mortem (Olson et al., 1986). According to earlier reports, myofibril fragmentation was a result of the tension created during post-mortem muscle contraction and the maximum fragmentation was observed as sarcomeres reached the length, 2.0-2.2 μm (Hattori and Takahashi, 1979). Later, it was proposed that myofibrillar fragmentation during the first few days of ageing was a result of degradation of cosatameres that link myofibrils to sarcolemma and N_2 lines that contains titin and nebulin (Taylor et al., 1995). Also, Z disk degradation is noted during ageing (Olson et al., 1986). It is thought that the Z filaments of Z disk are stabilized through lipids and the liberation of calcium-phospholipid compounds from Z disk leads to weakening of the structure (Shimada and Takahashi, 2003). In addition, rate of ageing is observed to vary with the muscle type; myofibrils in fast-twitch muscles degrade faster than that of slow-twitch muscles (Abbott et al., 1977).

In addition to the natural means discussed above, mechanical means can be employed to further increase tenderness. Electrical stimulation of the carcass immediately after slaughter is an accepted approach to overcome the post rigour shortening and associated toughening of meat (Rosenvold et al., 2008). The underlying concept is that electrical stimulation causes muscle spasm, accelerates glycolysis, induces muscle fibre rupture and thereby increases post-mortem

proteolysis which in turn decreases toughness (Hwang et al., 2003; Luoet al., 2008; Furguson et al., 2008). Also, tender-stretch processing is reported to produce tender beef through preventing acto-myosin bonding and thereby muscle shortening (Maher et al., 2005) and also through longer sarcomeres than conventionally suspended carcasses (Quarrier et al., 1972).

Cooking is reported to decrease meat tenderness, compared to that of raw meat. As cooking progresses from 40 to 75 ºC, the toughness of *sternomandibularis* and *longissimus* muscles is increased several fold. This toughening of meat between 40 and 50 ºC is attributed to decreased solubility of myosin (Davey and Gilbert, 1974; Cross et al., 1976). Meat from *semitendinosus* cooked above 85 ºC, is always tougher than those cooked at lower temperatures regardless of cooking time (Califano et al., 1997). In other research, as cooking temperature increases from 40 to 90 ºC, total juice loss is increased and expressible moisture content is decreased in *biceps femoris, deep pectorali, semitendinosus and semimembranosus* muscles (Bouton and Harris, 1972). However, others report that higher water holding capacity of meat proteins after cooking is related to decreased toughness. The liberation of water (specially at >pH 4.3) is related to increased toughness (Gault, 1985). Overall, it is clear that cooking introduces a toughening effect on myofibrillar proteins.

Among the other factors that determine tenderness/ toughness of beef, cattle breed is reported to have a significant effect on tenderness due to differences in myofibrillar fragmentation and the relative proportion of white fibres in muscles (Strydom et al., 2000). The breed effect is also evident between *Bos taurus* and *Bos indicus* where muscles from *Bos indicus* were tougher than that from *Bos taurus* breed (Norman, 1982). Also, differences in pre-slaughter handing of animals that leads to stress induced pH decline and post-slaughter handling of meat have an impact on the overall sensory tenderness (Dransfield et al., 1982; Harper, 1999).

2.7.2 Toughness of connective tissues

The contribution of connective tissue to toughness of meat is reported for different species of animals, for example, cattle (Swatland and Findlay, 1997; Swatland, 2006), swine (Fang et al., 1999) and chicken (Liu et al., 1996). However, the relationship between connective tissue and toughness of beef is controversial. As early as 1907, mechanically measured toughness of different cuts of beef is related to their connective tissue content (Lehmann, 1907). According to Nishimura et al. (1999), during fattening of 9-20 months old cattle, toughness increase is parallel to the perimysium thickness increase. However, between 20-32 months, toughness of beef is gradually decreased subsequent to the deposition of fat in muscles. Discrepancies are such that, another similar study reports that perimysium thickness of cattle (15-18 months old) has no relationship with shear force (Brooks and Savell, 2004). The contribution of connective tissue to meat toughness remains unclear and some argue that connective tissue has little impact on toughness but biophysical aspects of muscle fibres play a more important role (Newbold and Harris, 1972; Tornberg, 1996; Lepetit et al., 2000).

The relationship between collagen content and toughness has been extensively studied but the results are contentious and often contradictory. As reported by some authors, muscle collagen concentration is closely correlated to sensory tenderness (Kim et al., 1967; Young and Braggings, 1993; Rhee et al., 2004; Jurie, et al., 2007) and also to shear force, the mechanical index of tenderness (Torrescano et al., 2003). Nevertheless, some others report that total collagen concentration has no relationship to tenderness or shear force and collagen content varies greatly among *longissimus* muscles originating from cattle of similar age, gender, breed and quality grade (Maher et al., 2005; Delgado et al., 2005). Among muscles from three age groups, the highest amount of collagen is detected in veal muscles and muscles from cows and steers have similar amounts of collagen. Also, collagen content and shear force do not show a significant relationship (Wilson et al., 1954). This observation is confirmed later; no

relationship is built between total collagen content and animal age but toughness is increased with the advancing age of cattle (Reagan, 1976). It is also thought that the effect of collagen on toughness can be completely eliminated after exposure to high temperatures, in particular above 60 ºC (Lehmann, 1907 and Christensen et al., 2000). One of the limitations of the current approach is that, chemical determinants of connective tissue content are confined to total and soluble collagen of meat. The other limitation is that, observations on connective tissue driven beef toughness are often made through measurements collected while connective tissue are still embedded in meat. No previous research has studied the relationship between the quantity of connective tissue in a muscle and its tenderness/toughness.

Cross-linking in connective tissue is regarded as a contributor for the increased meat toughness in old animals (Marsh, 1977). According to Bailey (1985), multivalent collagen cross-links are formed as an animal matures. These cross-links develop the tension during heating (from 60-70 ºC) and compress muscle fibres to increase the amount of protein per unit area. Consequently, toughness of meat increases. An increase in pyridinoline cross-link content (moles/mole of collagen) of different muscles is reported with the increasing age of rats, but at different rates (Palokangas et al., 1992). Similar observations are made with bovine muscles, *longissimus dorsi, semitendinosus and extensor carpi ulnaris*, where it was shown that lysylpyridinoline and hydroxylysylpyridinoline cross-link contents (moles/mole of collagen) increased with the age of the animal (Bosselmann et al., 1995).

2.7.3 Toughness of epimysium

Epimysium is often served as part of a steak but direct measurement of toughness of epimysium was rarely reported in the literature. Field et al. (1969), has determined the shear force of beef cores from *longissimus* and *biceps femoris* containing epimysium to conclude that epimysium

cooked to 71.1 ºC has lower shear force values than that cooked to 60 ºC. Whiting and Strange (1990) have measured shear force of isolated epimysium from beef chucks using a Lee-Kramer multiple blade. In their study, thin epimysial strips (8.5 x 50 mm) were equilibrated for 15 min in water (control), 0.5 M hydrochloric, phosphoric, acetic and lactic acid, 0.15 M citric acid, 2 M sodium hydroxide and 8 M urea and then washed with water before determining shear force. The only treatments that reduced the shear force less than the control were 2 M sodium hydroxide and 0.5 M lactic acid. As such, many opportunities exist to investigate the epimysium driven toughness of meat.

2.7.4 Meat toughness as influenced by animal age

Age of the animal is generally regarded as a contributing factor for toughness of meat. Supporting this idea, Reagan et al. (1976) has shown that shear force of *longissimus* muscles from cattle of 10 months to 27 years old, significantly increased with the advancing age of animals. Also, Shorthose and Harris (1990) have shown that shear force values of twelve different muscles (that have undergone electrical stimulation or tender-stretch processing) from cattle of 1-60 months old increased with the advancing age. Because, myofibrillar shortening was prevented in this experiment, connective tissue was considered as the contributing factor for increased toughness. Age is reported to have a significant effect on shear force values of cow meat in the age groups of 2-4 years, 6-8 years and 10-12 years (Xiong, 2007). A linear relationship between age and toughness of meat is observed for pork and chicken (Fang et al., 1999; Iqbal et al., 1999). Shear force values of pork *semitendinosus*, from neonates to 6 months old pigs increased with age and this increase was significantly correlated with the thickness increase in perimysium (Fang et al., 1999). Also, a linear correlation was reported between toughness of broiler meat and the advancing age of hens. Because age correlates well with the accumulation of pentosidines (AGE cross-link) as well, it is thought toughness increase is resulting from increase pentosidines in meat (Iqbal et al., 1999). Mature forms of collagen

cross-links are believed to be a major cause of age related toughness of meat and it is discussed in section 2.7.2.

However, other research contradicts the reported linear relationship between advancing animal age and increasing shear force (Robertson et al., 1984; Li et al., 2007). Shear force values of *semimembranosus* muscles from young (24-29 months) and old (48-54 months) buffalo and steers are not related to the age of animals (Robertson et al., 1984). In another research, shear force values of *semitendinosus* muscles from cattle of 24-72 months are shown to increase with the age only up to animals reach full maturity at the age of 36-42 months but after body maturation, shear force increase become less significant due to increasing intramuscular fat (Li et al., 2007). According to Harper (1990), chronological age has little effect on determining meat toughness but other factors such as pre-slaughter stress that brings meat pH between 5.8-6.2, physiological age, duration on high-energy diets, nutrient deficiencies that lead to wasting of myofibrils at a higher rate than connective tissues, growth path (the pattern of live weight gain) during early life that modify the muscle structure and animal management practices such as castration have a significant effect on toughness/ tenderness of meat.

2.7.5 Meat toughness as influenced by marinade pH

A marinade is a liquid that softens meat and improves flavour. It is thought that marinades influence the final pH of meat to deviate from isoelectric point and thereby change the physicochemical properties (Oreskovich et al., 1992). These authors have altered the muscle pH with phosphate buffer to achieve pH values 3.75, 4.64, 5.62, 6.81, 8.39 and 9.00 and observe that muscles at pH 4.64 and 5.62 are the toughest. Meat pH higher and lower than the aforementioned range contributes to tender meat. Acetic and lactic acids are reported to solubilize connective tissue collagen of “restructured” beef that is products of mechanically debonned meat (Arganosa and Marriott, 1989). In other research, structural deformation is

observed in meat treated with 2% lactic and acetic acid mixture but without reduced toughness (Mikel et al., 1996). In addition, 1 and 1.5% lactic and citric acids are reported to lower the onset temperature of collagen thermal denturation from 64.9 to 63.4 ºC (Berge et al., 2001) and to 39 ºC (Aktas and Kaya, 2001). As the concentration of lactic and citric acid increase from 0.5 to 1.5%, shear force of longissimus muscles, marinated with acids, is considerably reduced (Aktas et al., 2003). Citrus juice is another marinade that lowered the pH of beef from 5.7 to 3.1 and reduces the shear force from 178 to 44 N/cm^2. Hamling et al. (2008 a) observe that enhanced beef steaks with a 20% solution of ammonium hydroxide, salt and carbon monoxide for 1, 7 and 14 days have increased shear force values with ageing. However, the enhanced steaks have shear force values lower than the controls. As a result of ammonium hydroxide treatment, beef steaks reached a maximum pH value of 6.7 (Hamling et al., 2008 b). In the experiments describe above, whole muscle is the target tissue and thus it is difficult to conclude the effect of those weak organic acids and alkali on connective tissue driven toughness. It is likely that at least some of the observed reduced shear force is resulting from myofibrillar protein solubilization and fragmentation. The effect of acids and alkali on skeletal muscle connective tissue is an area open for investigation.

2.7.6 Measures of meat toughness

Meat toughness is measured either by trained panellists or by mechanical means. However, meat toughness and /or tenderness is ultimately the consumer perception of quality influenced by both physiological and psychological response and also an amalgam of attributes such as resistance to biting, rubberiness, fibre cohesiveness, connective tissue amount, juiciness, chewiness and amount of residue immediately before final swallowing (Dransfield et al., 1984). However, not every consumer distinguishes toughness/tenderness of beef comprehensively, but is able to recognize differences among tough, intermediate and tender meat (Destifanis, et al., 2008). Texture attributes, scales, cooking conditions and level of training employed in taste

panel evaluation of tenderness can be inconsistent and that makes comparison of results across studies difficult (Sivertsen et al., 2002; Peachey et al., 2002; Voges et al., 2007; Destifanis et al., 2008). As consumers make the final decision of quality of meat, in particular tenderness, sensory evaluation is essential as a measure but the need for standardization of test methods is essential for meaningful comparisons.

A mechanical method to determine texture may be more appealing over sensory evaluation in its ability to process large number of samples in a limited time using cost effective procedures and without the interference of human perception (Janz and Aalhus, 2002). A standard test to determine meat texture needs to satisfy several requirements; (a) a strong foundation on known principles of tissue organization, physical forces and chemical composition, (b) universal applicability within raw and cooked skeletal muscles and (c) close agreement with sensory tests (Stanley and Swatland, 1976). Many attempts have been made over the years to develop an accurate instrumental method to measure texture (Rhodes, et al., 1972; Bouton and Harris, 1972; Dikeman et al., 1972; Caine et al., 2003) including efforts to employ computer imaging (Tan, 2004) and ultrasonic spectral feature analysis (Park et al., 1994). However, the Warner-Bratzler shear machine, developed by the combined efforts of Dr. Warner and Dr. Bratzler (Bratzler, 1949; Warner, 1952), is considered the most popular instrument so far despite the fact that physical attributes of meat that it measures is not well defined (Boccard et al., 1981). Warner-Bratzler shearing is also used to differentiate the force contributions from muscle fibres and connective tissues where the peak force is attributed to muscle fibres and the shoulder peak is attributed to connective tissues (Møller, 1981). A pictorial of these forces is shown in Figure 2.9.

Though popular, Warner-Bratzler shear measurements also have limitations, for example, not having common protocols for sample preparation and testing can lead to high variability in

results. Modifications in cooking method, end temperature and muscle core orientation also have introduced significant differences to shear values reported (Hedrick et al., 1968; Wheeler et al, 1994). In other work, variations in shear force data are observed with varying sample temperature at shearing. Repeatability of results is increased with the use of constant temperature of cooking but not with constant duration of cooking (Caporaso et al., 1978; Wheeler et al., 1996). Variations in shear force data are also observed among laboratories due to differences in protocols, execution of protocols and instrumentation (Wheeler et al., 1997). An attempt to streamline the protocol of Warner-Bratzler shear force determination is reported and accordingly, to ensure repeatability and reproducibility control is required during transport of live animals, slaughter, chilling of carcasses, post-mortem ageing before assessment, selection of location on muscle, freezing, heating, preparation of samples from heated meat and instrumentation (Boccard et al., 1981). Another international collaboration to establish "reference methods for the assessment of physical characteristics of meat" including Warner-Bratzler shear force is reported (Honikel, 1998).

As discussed already in the previous paragraphs, no method currently used for toughness/tenderness determination is free from flaw. Thus, the attention is drawn to establish a relationship between instrumental measures of tenderness and consumer perceived tenderness (Wheeler et al., 1997). In one such attempt, sensory tenderness ratings are observed closely related to peak shear force data (Seideman and Theer, 1986).

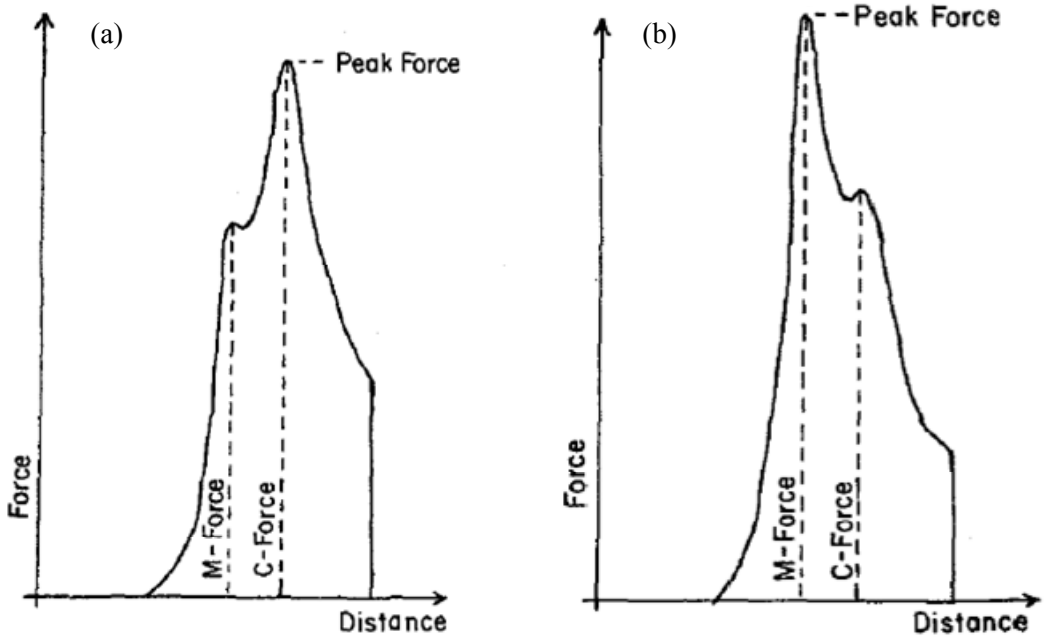


Figure 2.9 Force deformation curves for myofibrils and connective tissues. Warner-Bratzler shear force curves of bovine *semitendinosus* muscles (a) cooked at 60 ºC and at (b) 80 ºC. Peak force is the maximum force developed at shearing. M-Force is a result of myofibrillar proteins. C-Force is a result of connective tissue in meat. Figure 2 of Møller (1981) reproduced with kind permission from Elsevier Limited.

Threshold values of sensory tenderness/toughness reported in the literature are comparable. In one study, Warner-Bratzler shear force values (of steaks from beef top loin) of 4.6 and 3.9 kg are considered as slightly tender in retail and food service settings, respectively (Shackelford et al., 1991). In another study, 98% of consumers have considered shear force values <4.1 kg as tender for loin steaks (Huffman et al., 1996). The effect of environment on tenderness ratings also is studied; consumers are more concerned about tenderness in home setting than in a restaurant setting and threshold values reported for tenderness of loin, ribeye, bottom and top round is 4.6-5.0 kg in home and 4.3-5.2 kg in restaurant (Miller et al., 1995). In another study,

consumers have distinguished loin with shear force values <3.3 kg as very tender beef (Destifanis et al., 2008). The relationship between instrumental and consumer perceived tenderness is not as simple as reported above because taste panels are more sensitive in discriminating tenderness differences among muscles than instruments (Shackelford et al., 1995). In another study, neither taste panel nor instrument is able to discriminate tenderness differences between two tougher muscles *(Biceps femoris and semitendinosus)* but location has a significant effect on shear force data. This leads to the conclusion that shear force can be used to study tenderness differences between treatments within a muscle but not between different muscles (Shackelford et al., 1997). Consequently, shear force and taste panels are mostly used in a complementary manner, where shear force indicates differences caused by a treatment and taste panel indicates how such treatments are perceived by consumers (Janz and Aalhus, 2002).

3. INTRAMUSCULAR CONNECTIVE TISSUE PROPERTIES OF BOVINE *BICEPS FEMORIS*, *SEMIMEMBRANOSUS*, *GLUTEUS MEDIUS* AND *LONGISSIMUS* MUSCLES FROM HEIFERS AND COWS AND IMPACT ON SHEAR FORCE

3.1 Abstract

This study investigated the effect of muscle type and physiological maturity of animals on intramuscular connective tissue (IMCT) properties that are likely to have an effect on shear force. *Biceps femoris* (BF), *semimembranosus* (SM), *gluteus medius* (GM) and *longissimus* (L) muscles were excised from 6 cows (6±1 years) and 6 heifers (16±2 months). IMCT was isolated from muscles. Total collagen, Ringer's soluble collagen and shear force were determined for muscles. Thermal transition temperatures of muscle proteins and IMCT were determined. IMCT content significantly varied across muscles ($P<0.0001$) and maturity groups ($P<0.05$). IMCT had contributed to 3.9 to 11.75% of the dry weights of muscles investigated. Total collagen content had differed among muscles ranging from 2.2 –5.8% on a protein basis. The total collagen content of IMCT was stable among muscles and between cows and heifers (37.3-46.3% g collagen /100 g dry IMCT). Solubility of collagen expressed as a fraction of total collagen was evidently reduced in GM, SM and L muscles with the physiological maturity of animals but this phenomenon was not observed in BF muscles as solubility was already low in BF from heifers. The changes in shear force were not related to the changes in soluble collagen content. Shear force of BF, SM and L muscles was increased with the physiological maturity of the animal, but GM shear force was similar for either age group. This study demonstrated that shear force data of bovine BF, SM, and L could not be explained by the quantity of IMCT in muscles.

3.2 Introduction

Tenderness of beef has been identified as one of the key attributes that determine palatability of meat and thereby consumer satisfaction (Miller, 1995; Dikeman, 1987; Lorenzen et al., 2003; Feuz et al., 2004). It is also important to note that average consumers are able to discriminate among tender, intermediate and tough meats and are willing to pay a premium for tender steaks (Boleman, 1997; Lusk et al., 2001, Sivertsen, 2002). Huffman et al. (1996) reported that consumer perception of tenderness could be closely predicted by shear force measurements. Shear force based characterization of muscles was reported; nevertheless, results were not always comparable. Wheeler et al. (2000) compared the toughness of *biceps femoris*, *gluteus medius* and *semimembranosus* against *longissimus* from heifers and steers to conclude that all four muscles were either tender or intermediate in tenderness. On the contrary, Shackelford et al. (1995) reported that both bovine *semimembranosus* and *biceps femoris* from less than 18-month-old animals were tough, whereas *gluteus medius* and *longissimus* were intermediate and the most-tender muscles, respectively. Enhancing the complexity, Reuter et al. (2002) reported the location specific toughness differences within muscles. As such, there is a great eagerness to understand the reasons for toughness variations in beef muscles.

Both myofibril and connective tissue properties influence meat tenderness. Relationships were established between meat toughness and muscle fibre diameter and also between toughness and the size of the fibre bundle (Brady, 1937; Herring et al., 1965). Tornberg (1996) in a review presents the possible relationship between post-mortem muscle fibre contraction and toughness. Lehmann (1907) was the first to report the possible relationship between connective tissue and muscle toughness. Collagen is the most abundant connective tissue protein and often studied as an indicator of connective tissue in meat (Mitchell, 1928; Kim et al., 1967; Prost et al., 1975; Rhee et al., 2004). In some studies, positive correlations were observed between total collagen content and toughness of bovine muscles measured as shear force (Torrensco et al., 2003; Riley

et al., 2005), however in other studies, no difference was observed between total collagen contents of tough and tender muscles (Field et al., 1970; McKeith et al., 1985). Accordingly, no clear relationship was established between collagen measurements and toughness of muscles.

Age of the animal also was reported to influence the meat toughness; in beef loin muscles, a trend in increasing shear force was noted as the maturity grade (USDA) was increased (Romans et al., 1965; Parrish et al., 1979). Also, Warner-Bratzler shear force values of *semimembranosus, longissimus and triceps brachii* muscles from heifers were increased after 400 days of age (Zinn et al., 1970). Similarly, a decrease in tenderness was reported as the age of cattle increased from 10 months to 27 years (Reagan et al., 1976). However, most of the research on tenderness was conducted using meat from young animals because such meat was in high demand in retail markets. For example, Morgan et al. (1991) used eleven subprimals (blade, arm, chuck, strip loin, rib, tender loin, sirloin, round tip, bottom round, top round and eye of round) to conduct a survey on tenderness. Huffman et al. (1996) used loin steaks to determine tenderness of meat served at different settings. Miller et al. (2001) used strip loins of USDA select quality grade to determine consumer acceptability of meat. Shackelford et al. (1997) used *longissimus* muscles from steers <18 months old to establish criteria on tenderness. However, it has become increasingly important to investigate the tenderness attributes of meat from older animals since international trade of beef from mature animals in Canada was conditionally suspended after the reported cases of Bovine Spongiform Encephalopathy (BSE) (Sparling and Caswell, 2006; LeBlanc, 2007; Rude et al., 2007).

Efforts have been made to physically remove intramuscular connective tissue as a whole and also to differentiate perimysium and endomysium (McClain, 1969; Fujii and Murota, 1982; Light and Champion, 1984; Möller et al., 1993; Avery, 1995). Light et al. (1985) has quantified

endomysium and perimysium of *psoas major*, *longissimus dorsi*, *pectoralis profundis*, *sternomandibularis*, *gastrocnemius* and *semitendinosus* muscles from steers. However, no efforts were reported to characterize BF, SM, GM and L muscles from cows and heifers based on their intramuscular connective tissue contents and to demonstrate any relationship of the latter with the beef toughness. This study was carried out with the hypothesis that 'intramuscular connective tissue content in a muscle is an indicator of toughness', in order to differentiate selected bovine muscles from heifers and cows. Also, it was envisaged to understand the relationship among animal maturity, muscle type and shear force.

3. 3. Materials and Methods

3. 3.1 Meat sampling

This study used animals of two physiological maturity groups: 6 cows, Canada grade D2 with an average age of 6 ±1 years and average carcass weight of 331±64 kg and 6 heifers, Canada grade Y1 with an average age of 16±2 months and average carcass weight of 287±31 kg. Animals were slaughtered at a provincially inspected abbatoir. After chilling carcasses to 4 °C for 48 h, *biceps femoris* (BF), *gluteus medius* (GM), *longissimus* (L) *and semimembranosus* (SM) muscles from both sides of each carcass were excised. Muscles were stored at 4 °C for aging. On the 14th day post-mortem, after removing epimysium and fat, paired muscles from each animal, except for GM, were divided into anterior, posterior and middle sections and about 2.5 cm thick steaks were prepared for shear force determination. The dorsal part of GM was first removed along the fat seam, as the fibre direction of dorsal part was different from the rest of the muscle. The remaining muscle was halved perpendicular to the fibre direction to obtain anterior and posterior sections. A slice was removed from each section of paired muscles from an animal and these three slices were pooled for chemical analysis. Samples were separately

vacuum-packed and stored at -30 °C until used. Samples for chemical analysis were thawed to -1 °C and ground three times through a 3.175 mm orifice diameter plate before use.

3.3.2 Proximate analysis

Total moisture and crude protein were analysed according to AOAC 950.46 B and AOAC 981.10 (1990) methods, respectively. Meat pH was determined according to Koniecko (1979) with 20 g of ground meat. All analyses were carried out in duplicate.

3.3.3 Isolation of intramuscular connective tissue (IMCT)

Perimysium and endomysium was extracted together from muscles according to Fujii and Murota (1982) procedure and will be referred to as IMCT. The reagents were pre-prepared and stored at 4 °C and the IMCT extraction was carried out at 4 °C. Briefly, 200 g of ground meat was mixed with 5 volumes (v/w) of 10 mM Tris-maleate in 0.1 M KCl at pH 7.2 and homogenized in a Waring commercial blender (Waring Products, Torrington, CT, USA) for 1 min. The homogenate was transferred into a 2 L Pyrex beaker and stirred vigorously using a magnetic stir bar for 12 h. At the end of stirring contents were passed through a metal sieve (225 holes/cm^2) to collect the insoluble connective tissue. The aqueous phase was discarded. To further remove the myofibrillar proteins from the collected connective tissue it was mixed with 1 L of Hasselbach-Schneider solution (0.6 M KCl, 10 mM $Na_4P_2O_7$, 1 mM $MgCl_2$ and 0.1 M KH_2PO_4 at pH 6.4) and stirred for another 12 h. Insoluble connective tissue was collected as previously described. The extraction step with Hasselbach-Schneider solution was repeated three times. Connective tissue thus collected was mixed with 500 mL of 0.6 M KI in 0.06 M $Na_2S_2O_3$ and stirred for 12 h. The latter step was repeated twice. Insoluble connective tissue was collected as previously described. Chemical contaminants in connective tissue were removed by stirring with 500 mL of double de-ionized water for 2 h. The fraction collected was

a mix of perimysium and endomysium. The wet weight of IMCT was recorded and it was stored at -20 °C before the determination of moisture content, total collagen and endothermic transition temperature.

3.3.4 Total collagen

Total collagen contents of raw ground meat and oven dried IMCT (at 105 °C) were determined, with duplicates for each sample, according to the AOAC 990.26 (1990) procedure for hydroxyproline determination in meat and meat products. Briefly, about 4.0 g of ground meat or 0.4 g of oven dried IMCT was digested overnight with 3.5 M H_2SO_4 in an oven at 105±1 °C. The hot digest was diluted to 100 mL with de-ionized water. After filtering, an aliquot was stored at -20 °C. Before chemical analysis, liquid samples were thawed to room temperature and appropriate dilutions were prepared. A sample aliquot of 2 mL was mixed with 1 mL of 1.41% chloramine T (w/v) and incubated for 20 minutes at room temperature. Then it was mixed with 1 mL of colour reagent (10 g 4-dimethylaminobenzaldehyde, 35 mL of 60% perchloric acid (w/w) and 65 mL of 2-propanol) and heated at 60±1 °C for 15 min. At the end of heating samples were cooled to room temperature in a water bath. Absorbance was measured at 558 nm using a Genesys 5 UV-VIS spectrophotometer (Genesys 5 UV-VIS spectrophotometer, Milton & Roy Spectronic, Ivyland, PA, USA). Calibration standards were prepared using trans-4-hydroxy-L-proline. A conversion factor of 8 was used, as prescribed by AOAC 990.26 (1990), to convert hydroxyproline to collagen.

3.3.5 Soluble collagen

The insoluble collagen content of raw meat was first determined and the difference between total collagen and insoluble collagen was presented as soluble collagen. This modification was necessary since the AOAC 990.26 (1990) method was not sensitive to very low concentrations

of soluble collagen extracted from meat. The Hill (1966) procedure was used to remove soluble collagen from meat. In brief, about 6 g of ground meat was well mixed with 12 mL of ¼ strength Ringer's solution in 30 mL polycarbonate tubes. The mixture was allowed to reach 77±1 °C in a pre-heated water bath and then maintained at that temperature for 60 min. After cooling the tubes were centrifuged at 2800x*g* (Beckman JC-HC, Beckman Instruments, Palo Alto, CA, USA) and at 20 °C for 30 minutes to collect the pellet. The aqueous phase was discarded. The pellet was mixed well with 8 mL of ¼ strength Ringer's solution and centrifuged as previously described. The aqueous phase was discarded and the pellet was used for analysis of insoluble collagen according to the AOAC 990.26 (1990) as previously described. Calibration standards were prepared using trans-4-hydroxy-L-proline (Sigma-Aldrich, St Louis, MO, USA). A multiplication factor of 8 was used, as prescribed by AOAC 990.26 (1990), to convert hydroxyproline to collagen.

3.3.6 Endothermic transition temperatures

The endothermic transition temperatures of meat and IMCT proteins were determined using a DSC 2010, differential scanning calorimeter (TA Instruments, New Castle, Delaware, USA). About 10 mg of ground raw meat or wet IMCT was weighed into aluminium pans. The pans were hermetically sealed and were stored at 4 °C until used (within 12 h). The moisture contents of raw meat and wet IMCT were 73.5±1.3% and 85.4±2.5%, respectively and no extra moisture was added. Pans were first brought to 20 °C in the calorimeter and then heating continued at a constant rate of 3 °C/min from 20 °C to 100 °C. The temperature of each transition peak and total energy requirement for the transition were computed using the Universal Analysis 2000 software (TA Instruments, New Castle, Delaware, USA).

3.3.7 Shear force

Thawed steaks cut from the anterior, posterior and middle sections of muscles were grilled to an internal temperature of 71 °C (Garland ED-30B electric grill; Condon Barr Food Equipment Ltd, Canada). Cooked steaks were cooled to 25 °C and from each steak, 7-9 samples of 1.27x1.27x 2.54 cm were cut parallel to the fibre direction. The distinct band of connective tissue in GM muscles was eliminated from shear samples to improve structural uniformity. Samples were sheared perpendicular to the fibre direction using TMS-Pro texture system (Food Technology Corporation, Sterling, Virginia, USA) equipped with a Warner-Bratzler shear attachment. Maximum force generated during shearing was recorded as the shear force maximum. Shear force contributions from myofibres and from connective tissue were determined from the force-deformation curves of the Warner-Bratzler shear device according to Møller (1981). The height of the first peak was attributed to the myofibrils and the height of the second peak was assigned to the connective tissue**.**

3.3.8 Statistical Analysis

The PROC GLM procedure of SAS 8.1 programme (SAS Institute Inc, Cary, NC, USA) was used to analyse data. The model used to analyse the physicochemical properties of post-mortem muscles included the effects of maturity, muscle and a random term of animal nested within maturity. Means for both main effects and the interactions are provided. Mean separation was carried out using least significant differences (LSD). A separate analysis was conducted using the PROC GLM procedure of SAS 8.1 to compare the physicochemical properties of meat and connective tissue of each mucle from cows and heifers by considering data as originating from 8 different treatments.

3.4. Results

Protein (P<0.01) and moisture (P<0.0001) contents varied between GM, BF, SM and L muscles but not between cows and heifers (Table 3.1). SM and L muscles from cows and heifers had more protein than BF muscles. Muscle type and animal maturity had no effect on pH.

Table 3.1 Characterization of bovine skeletal muscles on protein, moisture and pH

	Heifer				Cow					
	BF	SM	GM	L	BF	SM	GM	L	P	LSD
Protein	21.1 ±0.32	22.2 ±0.16	21.4 ±0.81	21.8 ±0.16	21.1 ±0.24	21.8 ±0.24	21.2 ±0.24	21.7 ±0.32	0.007	0.6
Moisture	73.2 ±0.40	74.2 ±0.12	72.9 ±0.32	72.0 ±0.32	74.6 ±0.36	74.8 ±0.36	73.4 ±0.53	73.4 ±0.40	0.0001	1.1
pH	5.4 ±0.04	5.4 ±0.08	5.3 ±0.04	5.4 ±0.12	5.3 ±0.04	5.3 ±0.04	5.4 ±0.04	5.3 ±0.04	0.473 (NS)	-

Means±SE are presented. N=6.
BF-*biceps femoris*; SM-*semimembranosus*, GM-*gluteus medius*, L-*longissimus*.

3.4.1. Intra muscular connective tissue (IMCT)

After extraction from the meat matrix the moisture content of IMCT was 85.4±2.5% and which was higher than that of the intact tissue. To overcome relative differences in moisture contents, dry weights (at 105±2 ºC) of IMCT were determined and presented as dry IMCT g/ 100g raw weight of meat. IMCT content had significantly varied between cows and heifers (P<0.05) where meat from cows had more IMCT than meat from heifers (Table 3.2 and Table 3.3). Also, muscle type had a significant effect on IMCT content (P<0.0001). GM had the highest amount of IMCT and BF had the second highest amount of IMCT. SM and L muscles had similar amounts of IMCT. When computed on a dry matter basis *[(IMCT dry weight/ Meat dry weight)*100]* IMCT contents of GM was 9.4%. BF had 7.8% IMCT. SM and L muscles had 5.1% and 4.2% IMCT, respectively (Table 3.3).

Table 3.2 Probabilities of main effects and their two-way interactions of physicochemical properties of connective tissue and meat

Physicochemical property	Maturity	Animal (Maturity)	Muscle	Maturity*Muscle
Dry IMCT (g/100g raw meat)	P<0.0499	P<0.2719	P<0.0001	P<0.0646
Dry IMCT (g/100g dry meat)	P<0.0267	P<0.2723	P<0.0001	P<0.0771
Total collagen (g/ 100g dry IMCT)	P<0.8420	P<0.9719	P<0.5057	P<0.6043
Total collagen (g/100g raw meat)	P<0.0423	P<0.0124	P<0.0001	P<0.3457
Soluble collagen (g/100g raw meat)	P<0.0018	P<0.0939	P<0.0001	P<0.0006
Soluble collagen per Total collagen (%)	P<0.0002	P<0.0784	P<0.0604	P<0.0025
Peak I (°C)	P<0.9253	P<0.1049	P<0.8536	P<0.8651
Peak II (°C)	P<0.0195	P<0.1087	P<0.0095	P<0.0284
Peak III (°C)	P<0.7839	P<0.0330	P<0.6058	P<0.3516
Total energy for transitions (J/g)	P<0.1558	P<0.2654	P<0.5400	P<0.2124
Peak IMCT (°C)	P<0.6888	P<0.2551	P<0.0004	P<0.1825
Energy for IMCT transition (J/g)	P<0.6780	P<0.1750	P<0.0014	P<0.0632
Maximum shear force (N)	P<0.0001	P<0.1816	P<0.0001	P<0.0003
Myofibril driven shear force (N)	P<0.0007	P<0.3850	P<0.0001	P<0.1071
Connective tissue driven shear force (N)	P<0.0005	P<0.0293	P<0.0001	P<0.0225

BF-*biceps femoris*, GM-*gluteus medius* ,L- *longissimus,* SM- *semimembranosus.* N=6.

Table 3.3 Effects of animal maturity and muscle type on physicochemical properties of connective tissue and meat

		Physicochemical property							
		Dry IMCT (g/100g raw meat)	Dry IMCT (g/100g dry meat)	Total collagen (g/ 100g dry IMCT)	Total collagen (g/ 100g raw meat)	*Soluble collagen (g/ 100g raw meat)	*Soluble collagen on total collagen (%)	Peak I (ºC)	*Peak II (ºC)
Maturity	Cow	1.9±0.4	7.4±1.5	41.3±2.9	0.85±0.04	0.07±0.01	8.9±2.2	52.6±0.2	62.6±0.2
	Heifer	1.6±0.2	5.8±0.8	41.1±3.5	0.69±0.04	0.15±0.02	21.2±3.1	52.6±0.4	61.8±0.5
	P	0.049	0.027	0.842 (NS)	0.0.042	0.002	0.0002	0.925 (NS)	0.019
	LSD	0.289	1.092	-	0.083	0.028	3.077	-	0.449
Muscle	SM	1.3±0.1	5.1±0.5	38.1±3.1	0.57±0.04	0.09±0.02	16.0±3.3	52.5±0.2	62.8±0.3
	BF	2.0±0.1	7.8±0.5	41.4±3.0	0.86±0.10	0.10±0.03	12.0±3.0	52.6±0.2	61.7±0.4
	GM	2.5±0.4	9.4±1.5	43.6±3.8	1.11±0.10	0.18±0.04	17.9±4.7	52.7±0.2	62.0±0.3
	L	1.1±0.1	4.2±0.4	41.7±2.7	0.54±0.04	0.07±0.02	14.3±3.7	52.7±0.5	62.4±0.4
	P	0.0001	0.0001	0.505 (NS)	0.0001	0.0001	0.060 (NS)	0.853 (NS)	0.009
	LSD	0.41	1.54	-	0.12	0.04	-	-	0.64

Means±SE of parameters are shown above. N=6.
BF-*biceps femoris,* GM-*gluteus medius*, *BF*-biceps femoris*, GM*-gluteus medius, *L*- longissimus, *SM*- semimembranosus.
*Symbol indicates that a significant maturity*muscle interaction is observed. A mean comparison across muscles from cows and heifers is given in Table 3.4.
NS= Not significant

Table 3.3 continued……

		Physicochemical property						
		Peak III (ºC)	Total energy for transitions (J/g)	Peak IMCT (ºC)	Energy for IMCT transitions (J/g)	*Maximum shear force (N)	Myofibril driven shear force (N)	*Connective tissue driven shear force (N)
Maturity	Cow	72.5±0.4	2.3±0.1	61.3±0.9	2.2±0.2	95.7±13.6	80.3±8.8	85.3±15.7
	Heifer	72.4±0.4	2.4±0.1	61.0±0.8	2.3±0.4	66.3±6.3	61.3±4.8	52.5±8.9
	P	0.783 (NS)	0.155 (NS)	0.688 (NS)	0.678 (NS)	0.0001	0.0007	0.0005
	LSD	-	-	-	-	7.549	7.571	8.619
Muscle	SM	72.3±0.4	2.4±0.1	62.7±0.5	2.3±0.2	94.1±9.4	84.3±7.0	85.1±11.4
	BF	72.5±0.4	2.4±0.1	61.3±0.8	2.1±0.2	106.7±13.3	78.4±9.0	101.7±14.1
	GM	72.5±0.3	2.3±0.1	59.2±0.8	2.8±0.4	66.9±4.8	65.0±4.3	50.8±6.6
	L	72.7±0.3	2.3±0.2	61.3±0.8	1.8±0.2	56.2±6.2	55.2±6.2	37.9±5.8
	P	0.605 (NS)	0.540 (NS)	0.0004	0.001	0.0001	0.0001	0.0001
	LSD	-	-	1.4	0.49	10.68	10.72	12.19

Means±SE of parameters are shown above. N=6.
BF-*biceps femoris,* GM-*gluteus medius*, *BF*-biceps femoris*, GM*-gluteus medius, *L*- longissimus, *SM*- semimembranosus.
*Symbol indicates that a significant maturity*muscle interaction is observed. A mean comparison across muscles from cows and heifers is given in Table 3.4.
NS= Not significant

Except for GM, the rest of the muscles from cows and heifers had similar amounts of IMCT whereas GM from cows had more IMCT than GM from heifers. It was noted that the interaction maturity*muscle showed a trend in becoming significant for IMCT content (P=0.06) (Table 3.2).

3.4.2 Total collagen

Total collagen contents of muscles significantly varied between cows and heifers (P<0.05) where meat from cows had more total collagen than meat from heifers (Table 3.2 and Table 3.3). Also, total collagen content significantly varied with the muscle type (P<0.0001) (Table 3.2). Changes in total collagen and IMCT contents (on a raw weight basis) of muscles had followed the same pattern. Accordingly, GM muscles had the highest amounts of total collagen, followed by BF muscles. SM and L muscles had similar amounts of total collagen but less than that of GM and BF (Table 3.4). However, total collagen content of GM might not be indicative of that of a GM steak as the distinct band of connective tissue in GM is usually removed during portioning. The ratio of collagen to dry IMCT was a constant across muscles and between cows and heifers (Table 3.3). A high correlation was observed between IMCT and total collagen contents (r=0.89, P<0.0001) (Table 3.5). When total collagen is presented as a percentage of the total protein in each muscle *[(Total collagen g/Total protein g)*100]* collagen in GM, BF, SM and L from heifers contributed to 4.6, 3.6, 2.4 and 2.2% of the protein, respectively. Similarly, contribution of collagen to protein in GM, BF, SM and L muscles from cows was 5.8, 4.6, 2.8 and 2.7%, respectively.

3.4.3 Soluble collagen

The interaction, animal maturity*muscle had significantly influenced the soluble collagen on a weight basis (P<0.001) and soluble collagen presented as a percentage of total collagen (P<0.05)

Table 3.4 Mean comparisons for physicochemical properties of meat and connective tissue from cows and heifers

	Heifers				Cows					
	BF	SM	GM	L	BF	SM	GM	L	P	LSD
Dry IMCT (g/100g raw meat)	1.9±0.1	1.2±0.1	2.1±0.2	1.1±0.1	2.1±0.1	1.4±0.1	3.1±0.5	1.2±0.1	0.0001	0.58
Collagen in IMCT (g/ 100g dry IMCT)	39.5±3.5	37.3±3.6	46.3±3.7	41.1±3.2	43.3±2.6	38.9±3.0	40.8±3.8	42.3±2.5	0.691	-
Peak IMCT (°C)	60.4±0.4	63.1±0.4	59.7±0.7	60.9±0.9	62.3±0.9	62.4±0.5	58.8±1.0	61.7±4.7	0.006	2.2
Energy for IMCT transition (J/g moist IMCT)	1.9±0.2	2.4±0.2	3.2±0.4	1.8±0.1	2.4±0.2	2.3±0.3	2.4±0.2	1.8±0.7	0.002	0.67
*Soluble collagen (g/100g raw meat)	0.11±0.03	0.12±0.02	0.28±0.01	0.11±0.01	0.10±0.02	0.07±0.01	0.09±0.02	0.04±0.001	0.0001	0.06
*Soluble collagen/ Total collagen (%)	13.6±3.3	21.4±2.8	28.2±1.2	21.8±1.9	10.5±2.8	10.72.2	7.6±1.9	6.8±2.0	0.0001	6.4
Peak I (°C)	52.7±0.1	52.5±0.3	52.6±0.3	52.6±0.8	52.5±0.2	52.6±0.2	52.8±0.1	52.4±0.2	0.975	-
*Peak II (°C)	60.8±0.2	62.6±0.5	61.9±0.4	61.9±0.5	62.7±0.2	62.9±0.2	62.0±0.3	62.9±0.2	0.001	0.90
Peak III (°C)	72.6±0.2	72.4±0.4	72.1±0.4	72.6±0.4	72.5±0.5	72.1±0.3	72.8±0.3	72.8±0.3	0.605	-
Total energy for transitions (J/g raw meat)	2.5±0.1	2.7±0.1	2.2±0.1	2.4±0.1	2.3±0.1	2.2±0.1	2.3±0.2	2.3±0.2	0.250	-
*Maximum shear force (N)	78±4.2	76±0.2	63±3.8	47±3.5	135±7.3	112±7.5	71±5.7	65±6.5	0.0001	15.4
Myofibril driven shear force (N)	64±2.5	71±3.4	63±3.9	47±3.5	93±9.4	97±5.1	67±4.8	63±6.3	0.0001	15.1
*Connective tissue driven shear force (N)	76±4.9	65±5.7	42±4.1	27±1.6	128±12.0	105±9.4	59±7.2	49±4.7	0.0001	19.6

Mean±SE are presented. N=6.
BF-*biceps femoris,* GM-*gluteus medius*, *BF*-biceps femoris*, GM*-gluteus medius, *L*- longissimus, *SM*- semimembranosus.
* Symbol indicates parameters with significant maturity*muscle interactions**.**

Table 3.5 Pearson correlation coefficients for physicochemical properties of skeletal muscles from cows and heifers

	Total collagen	Soluble collagen	IMCT	Peak I	Peak II	Peak III	Energy meat protein	Shear force maximum	Shear force myofibrils	Shear force connective tissue	Peak I IMCT	Energy IMCT
Total collagen	1.0											
Soluble collagen	0.32*	1.0										
IMCT	0.89***	0.12	1.0									
Peak I Myosin	0.05	-0.02	0.08	1.0								
Peak II Collagen	-0.24	-0.37**	-0.25	0.17	1.0							
Peak III Actin	-0.005	-0.21	0.04	0.37**	0.02	1.0						
Energy meat protein	-0.01	-0.04	0.07	0.29*	-0.04	0.34*	1.0					
Shear force maximum	0.21	-0.22	0.19	0.02	0.22	-0.02	-0.12	1.0				
Shear force myofibrils	0.17	-0.19	0.14	0.04	0.24	-0.04	-0.18	0.90***	1.0			
Shear force connective tissue	0.25	-0.23	0.21	-0.009	0.19	-0.09	-0.07	0.94***	0.83***	1.0		
Peak I IMCT	-0.50***	-0.10	0.52***	-0.11	0.27	-0.17	0.0007	0.21	0.18	0.23	1.0	
Energy IMCT	0.33*	0.39**	0.19	-0.05	0.14	0.01	0.01	0.05	0.08	0.04	-0.07	1.0

* P<0.05, **P<0.01, ***P<0.0001. BF-*biceps femoris,* GM-*gluteus medius* ,L- *longissimus*, SM- *semimembranosus*.
N=48.

(Table 3.2). Soluble collagen contents of tested muscles were less than 0.3% of the weight of muscle (Table 3.4). Collagen solubility, on a total collagen basis, was significantly different across muscles from cows and heifers. The highest solubility, about 28% of total collagen, was observed in GM muscles from heifers (Table 3.4). However, soluble collagen content of GM might not represent that of a GM steak because the distinct band of connective tissue is often eliminated from steaks. For all muscles, except for BF, solubility of collagen was significantly decreased with the maturity of the animal (Table 3.4). The highest reduction in solubility was noted for GM and L muscles; solubility of GM from heifers was 28.2% and that from cows was 7.6%. Similarly, solubility of L muscles from heifers was 21.8% and which was decreased to 6.8% in cows. Also, solubility of collagen of SM from heifers (21.4%) was higher than that of cows (10.7%). Between soluble collagen and total collagen, a weak correlation was noted ($r=0.32$, $P<0.05$) (Table 3.5).

3.4.4 Endothermic transition temperatures

DSC thermograms showing endothermic transition temperatures for proteins of muscles investigated are given in Figure 3.1. Three endothermic transitional peaks were observed with means at 52, 62 and 72 and those were designated as peak I, II and III, respectively. The temperatures for peaks I and III were constant across muscles and also between cows and heifers (Table 3.2 and Table 3.3). Conversely, temperatures for peak II was influenced by the maturity*muscle interaction ($P<0.05$) (Table 3.2). Peak II temperatures of BF and L muscles from cows were significantly higher than that from heifers but peak II temperatures for SM and GM were not different between cows and heifers (Table 3.4). The termination temperatures of the thermal transition were consistent across all muscles and were in the range of 76.9 –77.9 °C (Table 3.6). The muscle (type) and maturity of animals had no effect on the energy requirement of the thermal transition of meat proteins (J/g of raw meat) (Table 3.2 and Table 3.3).

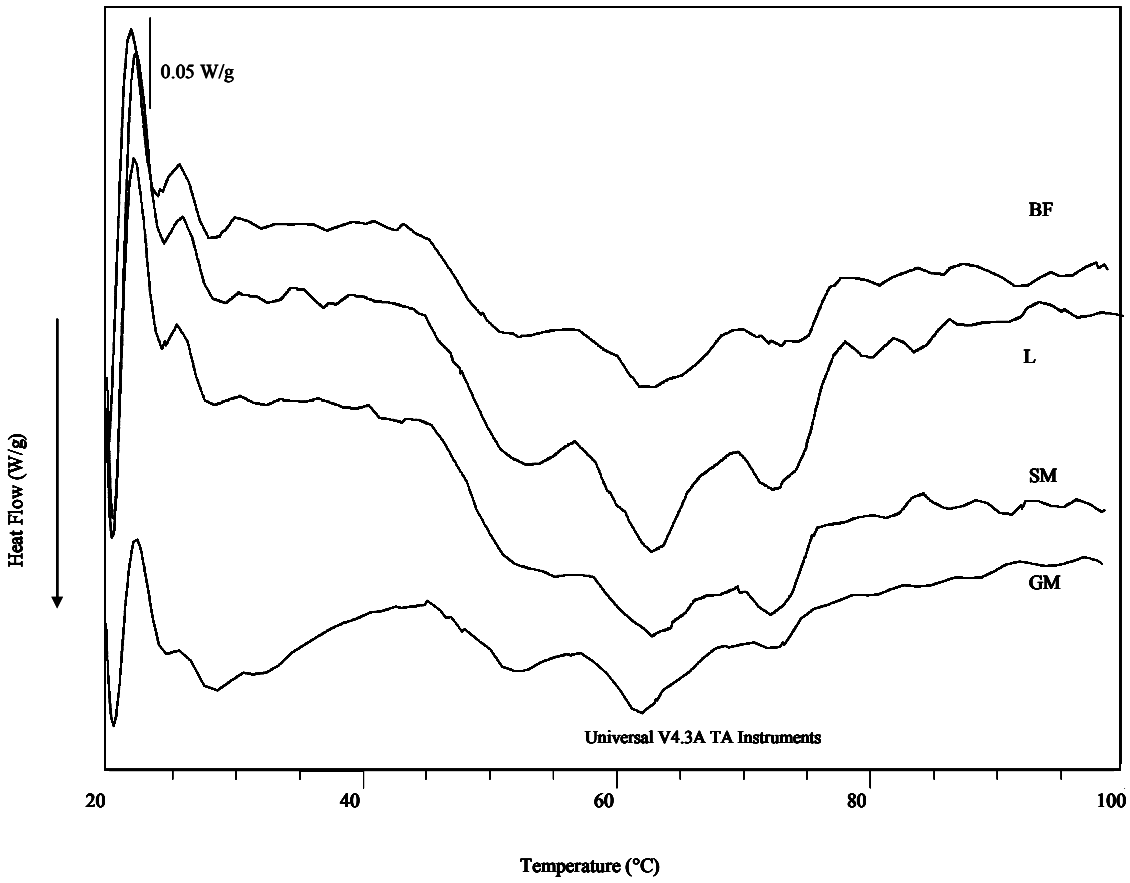


Figure 3.1 DSC thermograms of cow muscle proteins. BF-*biceps femoris*, L-*longissimus*, SM-*semimembranosus* and GM-*gluteus medius*. Moisture contents of muscles are listed in Table 3.1. Mean temperatures of peaks I, II and III are given in Table 3.3. and Table 3.4. The thermal transition onset and end temperatures are given in Table 3.6.

Table 3.6 Thermal transition temperature ranges of muscle and IMCT proteins

		Muscle		IMCT	
		Onset (ºC)	End (ºC)	Onset (ºC)	End (ºC)
Cows	BF	46.5±0.89	77.6±0.36	55.3±0.74	70.1±1.13
	SM	46.9±1.17	77.3±0.43	55.6±1.16	70.4±2.05
	GM	49.6±1.80	77.3±0.53	51.1±1.02	70.7±1.14
	L	47.5±1.23	77.9±0.41	55.8±1.20	71.2±1.67
Heifers	BF	49.0±1.73	77.9±0.43	53.6±0.71	67.2±1.54
	SM	47.4±1.17	77.8±0.45	55.6±0.62	70.4±0.95
	GM	48.2±1.58	77.8±0.72	54.1±1.13	67.8±1.09
	L	49.6±1.89	76.9±0.66	51.7±1.11	70.9±1.33

BF-biceps femoris, SM-semimembranosus, GM-gluteus medius and L- longissimus. Means±SE are presented. N=6.

Also, the above statement was true for the energy requirement of the thermal transition of meat proteins when computed on a protein basis (data not shown). The reported low values of energy for thermal transitions may be attributed to energy computed on the basis of the mass of the meat.

DSC thermograms for IMCT had produced a peak (Figure 3.2) in the range of 59.2 - 62.7 °C (Table 3.3). Giving due consideration to the fact that some of the properties of IMCT might have altered during the extraction process, it could be stated that the thermal transition temperature of IMCT was significantly influenced by the muscle type ($P<0.001$) (Table 3.2 and Table 3.3). Thermal transition temperature of IMCT from a selected muscle was similar between cows and heifers. The IMCT from SM, BF and L muscles had similar thermal transition temperatures. IMCT from GM muscles had shown the lowest thermal transition temperatures. The transition onset and end temperatures for IMCT were in the range of 51.1-55.8 °C and 67.2-70.9 °C, respectively (Table 3.6). This clearly showed that the thermal transition of most of the IMCT proteins (collagen) took place within the same temperature range where other meat proteins underwent thermal transition, even though that peak II temperature

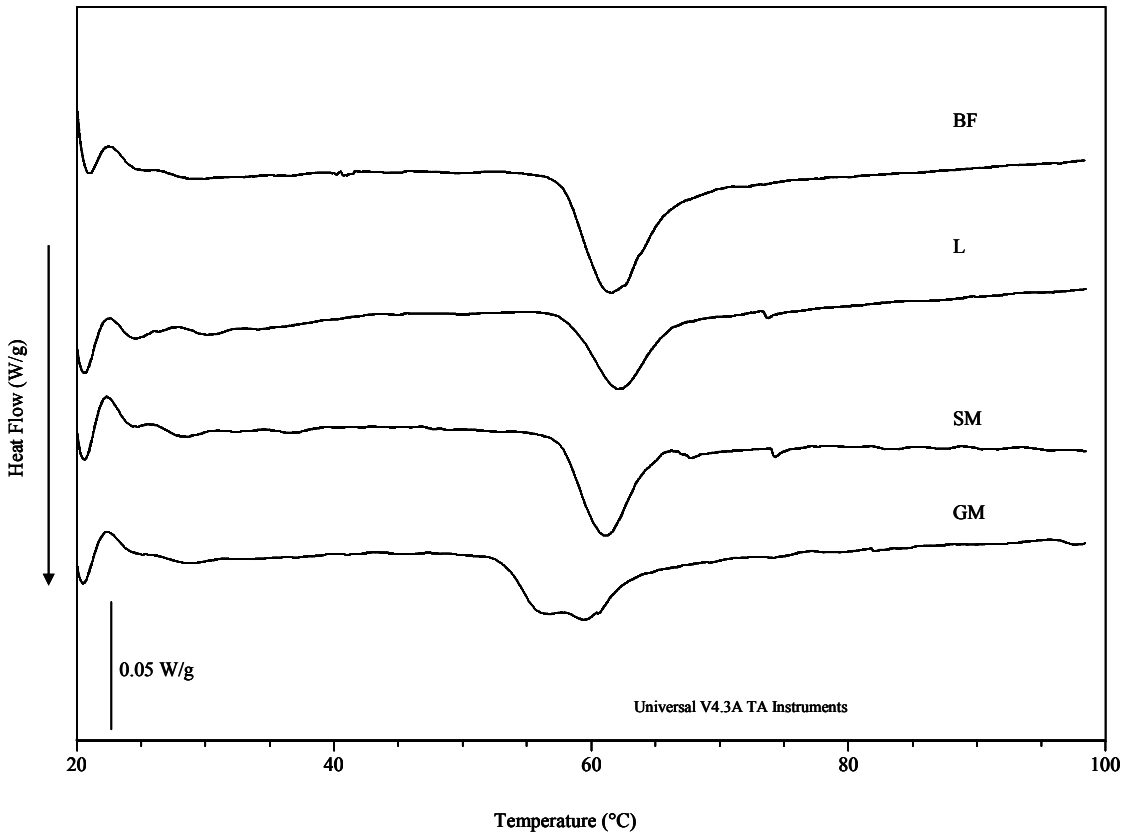


Figure 3.2 DSC thermograms of cow intramuscular connective tissue. BF-*biceps femoris*, L-*longissimus*, SM-*semimembranosus* and GM-*gluteus medius*. Average moisture content of IMCT was 85.4±2.5%. Mean temperatures of peaks are given in Table 3.3 and Table 3.4. The thermal transition onset and end temperatures are given in Table 3.6.

of meat and the peak of IMCT transition were similar. The interaction, maturity*muscle had shown a tendancy to have a significant effect on the energy requirement for the thermal transition of IMCT proteins (P=0.063) (Table 3.2). IMCT from GM muscles required more energy for the transition than all other muscles (Table 3.4), perhaps more bonds in GM were sensitive to thermal denaturation. Weak correlations were observed between energy (Table 3.4) used for transition of IMCT protein and soluble ($r=0.39$, $P<0.01$) and total ($r=0.33$, $P<0.05$) collagen contents in muscles (Table 3.5). The onset temperature of thermal transition of cow and heifer muscle proteins was in the range of 46.5–49.6 °C and had shown a considerable variation among muscles (Table 3.6).

3.4.5 Shear force

The highest force required for shearing muscle cores was reported as the maximum shear force. Myofibril driven shear force was the first peak and the connective tissue driven shear force was the second peak to appear on force deformation curves. The maximum shear force, which could be originating from both connective tissue or myofibrillar proteins, was significantly influenced by the maturity*muscle interaction ($P<0.001$). The connective tissue driven shear force was influenced by the interaction, maturity*muscle ($P<0.05$) (Table 3.2). The myofibril driven shear force was influenced by the animal maturity ($P<0.0001$) and muscle ($P<0.0001$) (Table 3.2 and Table 3.3). Between cows and heifers, myofibrils had contributed to the shear force maximum values of GM and L muscles but connective tissue had contributed to the shear force maximum values of BF muscles (Table 3.4). Shear force maximum of SM muscles from heifers was contributed by myofibrils but that of cows was contributed by both myofibrils and connective tissues. On the other hand, out of all muscles studied, the highest values for connective tissue driven shear force were observed for BF and SM from cows. The lowest values for connective tissue driven shear force were observed for L from heifers. GM and L muscles from cows had similar values connective tissue driven shear force. L from heifers had

lower values of connective tissue driven shear force than GM from heifers (Table 3.4). Based on the shear force maximum values, meat from cows had higher shear force values than that from heifers except for GM, where meat from cows and heifers had similar shear force values. However, it was noted that the reported shear force values for GM muscles could be lower than the actual because the thick band of connective tissue in GM muscle was eliminated from samples to attain structural uniformity. No significant correlation was observed between shear force values and any other physicochemical characteristic determined when data from GM muscle were included in the analysis (Table 3.5). Because a visible band of connective tissue was removed from GM before shear force determination, a second correlation analysis was carried out without data from GM (Table 3.7). Accordingly, shear force maximum ($r=0.68$, $P<0.0001$), shear force myofibrils ($r=0.49$, $P<0.05$) and shear force connective tissue ($r=0.72$, $P<0.0001$) were significantly correlated with total collagen content. Also, shear force maximum ($r=0.59$, $P<0.0001$), shear force myofibrils ($r=0.35$, $P<0.05$) and shear force connective tissue ($r=0.60$, $P<0.0001$) were significantly correlated with IMCT content.

3.5 Discussion

According to Light et al. (1985), perimysium and endomysium from bovine L muscles had contributed to about 2% of the dry weight of the muscle. In the present study, the amount of IMCT extracted from L muscles using Fujii and Murota (1982) extraction procedure were about 4.1±0.9% of the dry weight of muscles. This amount of IMCT was twice as high as the amounts reported by Light et al. (1985). Total collagen content in IMCT (100g) was computed as *(Total collagen in 100 g raw meat/ IMCT in 100 g raw meat)*100]*. Accordingly, IMCT from BF, SM, GM and L from heifers contained 40, 44, 47 and 44% collagen, respectively and IMCT from BF, SM, GM and L muscles from cows contained 46, 44, 40 and 48% collagen, respectively. Comparison of these data against data from the direct measurement of collagen in IMCT (g/100

Table 3.7 Pearson Correlation Coefficients for physicochemical properties of skeletal muscles from cows and heifers excluding GM muscle

	Total collagen	Soluble collagen	IMCT	Peak I	Peak II	Peak III	Energy meat protein	Shear force maximum	Shear force myofibrils	Shear force connective tissue	Peak I IMCT	Energy IMCT
Total collagen	1.0											
Soluble collagen	0.26	1.0										
IMCT	0.74***	0.07	1.0									
Peak I Myosin	-0.04	0.02	-0.003	1.0								
Peak II Collagen	0.16	-0.47*	-0.20	0.19	1.0							
Peak III Actin	0.04	-0.13	0.09	0.35*	0.007	1.0						
Energy meat protein	-0.10	0.09	-0.03	0.33*	0.01	0.32	1.0					
Shear force maximum	0.68***	-0.07	0.59***	0.04	0.22	-0.06	-0.25	1.0				
Shear force myofibrils	0.49*	-0.10	0.35*	0.05	0.27	-0.09	-0.31	0.89***	1.0			
Shear force connective tissue	0.72***	-0.03	0.60***	0.03	0.20	-1.3	-0.22	0.95***	0.85**	1.0		
Peak I IMCT	-0.16	-0.03	-0.16	-0.01	0.38*	-0.18	0.01	0.13	0.22	0.06	1.0	
Energy IMCT	0.24	0.14	0.11	-0.13	0.15	0.04	0.002	0.32	0.30	0.31	0.10	1.0

P<0.05, **P<0.01, ***P<0.0001. BF-*biceps femoris,* GM-*gluteus medius*, L- *longissimus*, SM- *semimembranosus*.
N=36.

g dry weight) (Table 3.3) showed the efficiency of intramuscular collagen extraction by Fujii and Murota (1982) method was in the range of 84-102%. It was noted from the data that IMCT content varied with muscle type and physiological maturity of animals (Table 3.2). IMCT (dry) contents of muscles from cows and heifers had varied between 4.1- 9.4% of the dry weight of muscles. Observed muscle specific differences in IMCT contents might be related to the functional needs of skeletal muscles as explained by Garcia-Bunuel and Garcia-Bunuel (1967), Kovanen et al. (1984) and Purslow (2005). Accordingly, slow twitch muscles that support the posture on a regular basis have more collagen than fast twitch muscles that support voluntary movements. In the muscles investigated, collagen had contributed to 37.3-46.3% of the dry weight of IMCT. On the other hand, more than the 50% of the dry weight of IMCT was non-collagenous of origin.

The amount of total collagen present in a unit weight of IMCT *[(Collagen g/IMCT g)*100]* on dry matter basis was consistent across muscles from cows and heifers and thus collagen content is a good indicator of amount of connective tissue present in a muscle. On the other hand, collagen had contributed to less than 6% of the wet weight of proteins in any of the muscles investigated. Meat from cows had more total collagen (on a weight basis) than meat from heifers. Also, meat from GM muscles had more total collagen than meat from other muscles. Meat from BF had more total collagen than SM and L muscles whereas SM and L had similar amounts of total collagen. Prost et al. (1975) had measured the total collagen content in bovine muscles from animals of 1-5 yrs of age to characterize them based on connective tissue content and reported that 3.34% of proteins in BF muscles were collagen. These values were similar to the values reported in the present study, where, collagen content of BF muscles from heifers and cows were about 3.6% and 4.6% of protein, respectively. According to Torrescano et al. (2003), BF from young bulls contained more total and insoluble collagen than GM from young bulls. In the present study, BF muscles had contained less total collagen than GM muscles and collagen

from heifer BF muscles were more insoluble than collagen from heifer GM muscles. Comparison between heifers and steers was acceptable according to Prost (1975) where sex of the animal had no effect on the total collagen content of muscles. Herring et al. (1967) compared bovine SM and L muscles and reported that SM had more total and insoluble collagen than L muscles. In a muscle profiling study, Von Seggern et al. (2005) had reported total collagen values for GM (1.5%), BF (1.0%), SM (0.6%) and L (0.4%) muscles, which were similar to those reported in this study.

In the present study, collagen from GM and L muscles from cows was three times less soluble than collagen from heifers when expressed as a percentage of total collagen *[(soluble collagen g/ Total collagen g)*100]*. Collagen from SM muscles from cows was two times less soluble than collagen from heifers. Remarkably, solubility of collagen from cow and heifer BF muscles was not very different from each other. Clearly, collagen from BF muscles from heifers was less soluble than collagen from SM, GM and L muscles from heifers. This indicated that in some muscles age related changes had reduce the solubility of collagen but in others, functional differences had reduced the collagen solubility regardless of maturity. Herring et al. (1967) reported that solubility of collagen in SM and L muscles was reduced with the maturity of animals in USDA grades A, B and E. According to Goll et al. (1964) collagen isolated from BF muscles of calves and steers were more soluble than those isolated from cows at 100 °C. The tendency of collagen to become less soluble with advancing age was observed with different species of animals: solubility of ovine collagen was reduced with the increasing age of the animal (Young et al., 1993) and a significant reduction in solubility of collagen was observed with the advancing age of cattle, sheep and pigs (Hill, 1966).

Martens et al. (1982) proposed that thermal transition of myosin, collagen and actin occurs within 40-60 °C, 56-62 °C and 66-73 °C, respectively. Endothermic transition peaks for three

main structural proteins of bovine muscles were re-established by Findlay et al. (1986) but with a higher peak temperature for actin, 81 °C. The temperature peaks for thermal transition of isolated collagen from bovine SM and L were 70.6 °C and 71.3 °C, respectively, and higher than values reported by others (McClain et al., 1972). Considering previously reported values, it was thought that three temperature peaks observed in DSC thermograms from meat of the present study were originated from myosin (peak I), collagen (peak II) and actin (peak III). It is note worthy that, temperature of peak I (myosin) and peak III (actin) of the present study were consistent across muscles and between cows and heifers. This might be an indication of the biochemical similarity of those proteins in different muscles and also from cows and heifers. Interestingly, temperature values for peak II (collagen) had differed among muscles. Also, values for peak II of BF and L muscles from cows were higher than those from heifers indicating high thermal stability of collagen from those muscles. Thermal stability of collagen from cow and heifer GM muscles was not different according to peak II temperature data. The temperatures for peak II of GM (61 °C), SM (62 °C) and BF (62 °C) muscles, reported by Torrensco et al. (2003), were similar to the temperatures reported for peak II in the present study. DSC thermograms of isolated IMCT had produced a single peak between 58.8- 63.1 °C with transition onset and end temperatures within the range of 51.1-55.8 °C and 67.2-70.9 °C, respectively. This clearly shows that transition temperatures of IMCT protein (collagen) overlaps the thermal transition temperature range of other meat proteins, for example, onset between 46.5–49.6 °C and end between 76.9 –77.9 °C. As peak II temperatures of meat proteins were similar to the peak temperatures of IMCT, it was assumed that thermal transition at 60-63 °C represented connective tissue proteins. The subtle differences in temperatures observed between peak II values of meat and the peak of IMCT might be attributed to the modifications made to the connective tissue matrix during the IMCT extraction. Stuart (1991) had reported that the enthalpy for meat protein thermal transition was 2 J/g and which is close to

the observations made in the present study (2.2-2.7 J/g). The energy utilization for thermal transition of meat proteins was similar between cows and heifers and among muscles.

The maximum force applied in shearing meat samples is the commonly reported shear force value. The relative contribution of myofibrils and connective tissue on shear force was seldom reported in the literature. As observed in this study, for BF, SM and L muscles, meat from cows had higher maximum shear force values than meat from heifers. When the distinct connective tissue band was removed from GM muscles, the maximum shear force values of meat from cows and heifers were not different (Table 3.4). The contribution of myofibrils of GM and L muscles to the maximum shear force was greater than that of connective tissues. The contribution of connective tissue in BF muscles to the maximum shear force was greater than that of myofibrils. In SM muscles, shear force contribution of myofibrils and connective tissue had differed with the maturity of animals where shear force values for meat from heifers were mostly contributed by myofibrils but shear force values for meat from cows were equally contributed by myofibrils and connective tissues. On the whole, BF and SM muscles from cows had higher shear force (maximum) values than other muscles studied. GM (after the distinct connective tissue band was removed) and L muscles from cows had maximum shear force values similar to that of BF, SM and GM from heifers. The lowest maximum shear force values were reported for L muscles from heifers. Shear force maximum, shear force connective tissue and shear force myofibrils had shown a positive correlation with total collagen and IMCT contents of BF, SM and L muscles (Table 3.7). Despite the observed correlations, the maximum shear force and connective tissue driven shear force of muscles did not follow the same pattern as IMCT of those muscles (Table 3.3 and Table 3.4). Therefore, it was thought that the amount of IMCT was not a useful predictor of shear force of muscles.

The effect of animal age on shear force values was scarcely reported in the literature. Using seven different muscles from veals, young cattle and adult cattle, Prost (1975) demonstrated that

shear force values of meat increase with the animal maturity. Shorthose and Harris (1990) made similar observations using twelve bovine muscles (which included BF, SM, GM and L from animals from 1-60 months old), accordingly shear force values increased with the advancing age of the animal. McKeith et al. (1985) had ranked thirteen different muscles from steers based on sensory properties and shear values and reported that BF was among the toughest muscles that generate a sense of high connective tissue during chewing. Further, GM was ranked as a moderately tough muscle with a low sense of connective tissue. Field et al. (1970) had compared properties of cow BF and L muscles to show that BF was twice as tough as L and BF contained 25% less labile collagen than L muscles. Bouton and Harris (1972) further verified the toughness of BF muscles from steers showing that shear force was not reduced in BF muscles after cooking for 8 hrs at 70 °C. In contrast, Rhee et al. (2004) reported that SM muscles from cattle of 14-16 months age was tougher than BF muscles but Torrensco et al. (2003) reported BF as a tougher muscle than SM in 16 months old bulls. According to Miller et al. (2001), the transition of consumer perception from tender to tough beef occurred between shear force values of 43-49 N. Consumer threshold values for tenderness reported by Huffman (1996) were similar, 41 N. Provided that the Huffman and Miller determined threshold values were valid, L from heifers were the only tender muscles observed in this study.

As observed in this study, no logical relationships were evident between shear force and soluble collagen of muscles. Shear force values of cow meat were higher than those of heifer meat except for GM, but soluble collagen did not show the same trend. Meat from cows had higher total collagen than meat from heifers. Maher et al. (2005) did not observe any relationship between collagen content and toughness of muscles. Shimokomaki et al. (1972) measured the sodium borohydride reducible collagen cross-links in bovine muscles from 1-15 yrs old cattle and argued that total collagen content in a muscle was not important but the amount of reducible collagen was important to determine the toughness of muscles. According to this study, IMCT

content varied with the maturity of animals and muscle type but did not always follow the same trend as shear force.

3.6 Summary and conclusions

The present study had investigated the effect of the maturity of animals and muscle type on intramuscular connective tissue properties to characterize selected bovine muscles and to establish a relationship between shear force and intramuscular connective tissue. It was evident from this work that the muscle type and physiological maturity of animals determines the amount of intramuscular connective tissue in a muscle. On a dry matter basis, GM muscles had the highest amount of intramuscular connective tissue (9.4%) and L and SM muscles had the least amounts, 4.1 and 5.1%, respectively. As collagen to IMCT ratio was invariable among muscles and also between cows and heifers, total collagen was a good indicator of connective tissue content of muscles. It was noted that collagen had contributed to less than 50% of the dry weight of intramuscular connective tissue and less than 6% of the muscle proteins. Collagen from cows was less soluble than collagen from heifers except for BF (on the basis of total collagen) and particularly collagen from cow BF muscles was less soluble than collagen from cow GM and L muscles. Solubility of collagen (on the basis of total collagen) from heifer BF muscles was similar to that of cows' and this indicated that the decreased solubility of collagen, regardless of physiological maturities evaluated, was a functional response of that muscle. Soluble collagen contents of muscles were not related to other parameters measured including shear force. DSC thermograms of isolated intramuscular connective tissue had confirmed that peak II temperatures observed in meat thermograms were resulting from intramuscular connective tissue proteins. The thermal transition of intramuscular connective tissue took place within a wide temperature range, with transition onset and end temperatures at 51.1-55.8 °C and 67.2-70.9 °C, respectively. Shear force values were increased in BF, SM and L muscles with the maturity of animals. Total collagen and IMCT contents of BF, SM and L muscles were

positively but moderately correlated with shear force indices. Because of the lack of a logical relationship among shear force and IMCT content, IMCT was not a true predictor of shear force.

3.7 Connection to the next study

It was observed in the preceding study that the thermal transition of collagen from perimysium and epimysium, from native to denatured state, had occurred within 51-71 °C. It was also noted that amount of collagen that become soluble had no relationship to toughness of muscles. Snowden & Weidemann (1978) reported that heat-treated tendons digested with enzyme pronase releases amorphous collagen leaving un-denatured collagen fibres suggesting that only part of the denatured collagen were released and the rest remained attached to the matrix. Combining the above observations, it was hypothesized that the degree of conversion of collagen from the native to denatured state (amorphous state) determines the degree of connective tissue driven toughness of muscles. However, studying the thermal properties of intramuscular collagen embedded in the muscle matrix was a challenge and it was deemed necessary to investigate isolated but intact connective tissue. The endomysium encircles individual muscle fibres and perimysium covers a group/s of muscle fibres. Their distribution at the microscopic level made physical removal extremely difficult while maintaining workable dimensions. Therefore, it was decided to use epimysium that encloses a whole muscle, in the following study as a model that demonstrates the behaviour of connective tissue during thermal processing. The rationale of selection of epimysium to replace intramuscular connective tissue is that collagen is the main protein in all of these tissues and more specifically they each contain collagen type III and I (Light et al., 1985).

4. DYNAMICS OF BOVINE EPIMYSIAL PROPERTIES SUBJECTED TO AQUEOUS HEATING

4.1 Abstract

This study was designed to understand the changes in the physicochemical properties of bovine *longissimus* epimysium during aqueous heating and also to envisage any relationship between shear stress and amorphous collagen. Epimysium from cows and heifers were subjected to heating at 55, 70, 80 and 95 °C for varying lengths of time. Properties measured included weight gain, thickness change, thermolabile and amorphous collagen produced and thermal sensitivity of collagen cross-links pyridinoline and Ehrlich chromogen (EC) as indicators of weakened epimysial structure. Different combinations of times and temperatures (70, 80 and 95 °C) had produced similar outcome in shear stress (force/area), weight gain, thickness change and also amount of thermolabile and amorphous proteins. Changes to epimysial properties occurred slowly when heated at 55 °C and shear stress values observed after 24 h heating at 55 °C were closer to those observed after 5 min heating at 70 °C. Thermolabile proteins consisted of single and double chains of collagen. After heating at 70 °C, EC and pyridinoline cross-links were released from epimysial matrix. The lowest shear stress values for heifer epimysium (2.1±0.7 N/mm^2) were reached at 70 °C. Heating to temperatures beyond 70 °C had decreased shear stress of cow epimysium but other properties changed little beyond that seen at 70 °C. Amorphous collagen resulting from heat induced phase transition was not influenced by the animal maturity and also not related to shear stress reduction.

4.2 Introduction

Tenderness is one of the most important quality attributes of beef that consumers consider (Boleman et al., 1997; Neely et al., 1998). Because connective tissue has long been considered as a contributor to beef toughness, research has focused on reducing the impact of connective tissue on the quality of beef (Nishimura, 1995; Christensen et al., 2000; Swatland, 2007). Beef toughness varies extensively between carcasses and also between and within muscles (Alsmeyer et al., 1965; Smith et al., 1969; Prost et al., 1975; Uytterhaegen, 1994; Shackelford et al., 1995). Also, the age of the animal is reported to influence the toughness differences, especially in connective tissue rich muscles (Shorthose and Harris, 1990; Huff and Parrish, 1993).

Exposure to high temperatures under moist or dry conditions is the best-known way to prepare meat. The cooking temperature should be appropriate to the connective tissue contents of muscles because it is observed that cooking to a low temperature (61 ºC) is sufficient to tenderize *longissimus* but cooking to a high temperature for long time (100 ºC for 25 min) is required to tenderize connective tissue-rich *biceps femoris* (Cover et al., 1962). It is also reported that moist heat cookery (57-62 ºC) can reduce the toughness of bovine *biceps femoris* more than grilling (Obuz et al., 2003). However, this observation was not widely accepted. Shear force values of meat from *semitendinosus*, prepared using four different cooking methods (braising, roasting, oven broiling and microwave) are reported to be similar (McCrae and Paul, 1974). Rate of heating is also known to influence toughness/tenderness (Bayne et al., 1969). According to the authors, meat roasted for a long time at low oven temperature (93 ºC) to reach an internal temperature of 67 ºC is tenderer than that roasted for a short time at high oven temperature (149 ºC) to reach an internal temperature of 70 ºC. However, according to another report the final temperature of cooking is more important than the rate of heating in determining tenderness of cooked meat (Laakkonen et al., 1970). The response of connective tissue to heating temperature was explained differently by different researchers. According to Davey and

Gilbert (1975), shortening of connective tissue during heating at 60-80 °C contributes to toughness increase in beef. According to Bouton and Harris (1972), and Christensen et al. (2000), connective tissue has an influence on toughness when meat is cooked to temperatures below 50 °C. As such, there is an ongoing debate on the actual contribution of connective tissue to beef toughness (Møller, 1980; Aktas, 2001; Brooks et al., 2004).

Collagen, a major connective tissue protein, is thought to undergo phase transition during heating with a change from crystalline state to an amorphous state (random coils). This new state is assumed to be resulting from flexibility of chains amid the rigid intermolecular bonds. The temperature of this transition is designated as the melting point (Garret and Flory, 1956; Flory and Garret, 1958). Collagen from cow epimysium is reported to melt between 58-68 °C with a peak at 63.3 °C (Field et al., 1970) and this temperature is known to be influenced by the moisture content of the sample, for example, the lower the moisture content the higher the transition temperature is (Rochdi et al., 1999). According to Martens et al. (1982), intramuscular collagen undergoes phase transition between 52-62 ºC. Another study reports the transition temperature as 68 °C (Bernal and Stanley, 1987). Does that imply that connective tissue collagen loses its impact on toughness beyond 68 °C? Miles et al. (1995) reported that thermal denaturation temperature of collagen was not a unique property and is dependant on DSC scanning rate, which was contradictory to the earlier explanations of Garret and Flory (1956) and Flory and Garret (1958). Thus, longer stabilization times at a lower temperature could denature collagen. Later, the same argument was reiterated by Wright and Humphrey (2002) who stated that the phase transition temperature of collagen "is neither a specific, thermodynamically defined temperature for the denaturation of collagen, nor a property of the material but the same degree of denaturation of collagen can be achieved through different combinations of temperature and rates of heating". The relationship between cooking

temperature and phase transition of collagen and their contribution to shear force reduction are yet to be clarified.

Heat stability and tensile strength of collagen from mature animals is generally attributed to multivalent, inter and intra molecular cross-links of collagen formed with the maturity of animal (Bentley, 1979; Bailey, 1989; Smith and Judge, 1991; Oxlund, et al., 1995). After a comprehensive analysis of porcine perimysial collagen cross-links, hydroxylysinorleucine, dihydroxylysinonorleucine, histidinohydroxymerodesmosine, hydroxylysyl-pyridinoline, histidino-hydroxylysinonorleucine and pyrrole cross-links, Avery et al. (1996) states that the nature of intermolecular cross-links (moles/ moles of collagen) are not related to shear force and (sensory panel determined) toughness of muscles. In other research, extremely tough bovine *sternomandibularis* muscle is found to contain less dihydroxylysinohydroxynorleucine, a highly thermostable collagen cross-link, than relatively tender muscles (Shimokomaki et al., 1972). After measuring the total cross-link content in intact tissue, they find a correlation between toughness and cross-links assuming cross-links are unequivocally thermostable. An oversight of their work is the lack of understanding the true response of cross-links to heating.

Based on electron micrographs of isolated collagen molecules first heated and subsequently hydrolysed with pronase, Snowden and Weidemann (1978) demonstrates that heat induced amorphous collagen remained attached to the matrix but pronase can liberate those amorphous regions without hydrolysing the non-amorphous, "native like", areas. Therefore, it is hypothesised that 'the degree of conversion of collagen from native to amorphous state during heating determines the degree of connective tissue driven toughness' and it is tested in this original study. This work also investigates the influence of heating time and temperature on bovine connective tissue properties including shear force (the first detailed study) and the impact of animal maturity on connective tissue properties. Epimysium is selected as the 'model

experimental material' to test the hypothesis. This decision is made to overcome the problems associated with excision of intramuscular connective tissue embedded in the muscle matrix. Epimysium is considered to closely represent intramuscular connective tissue as both contained type I collagen as the dominant species and is generally consumed as part of a loin steak. As indicators of weakened epimysial structure, thickness increase, shrinkage, weight gain, protein release, pronase liberated amorphous protein, EC and pyridinoline cross-link contents and shear force are measured after exposing the tissue to temperatures below and above the thermal denaturation temperature of collagen.

4.3 Materials and Methods

4.3.1 Sample preparation and aqueous heating

Paired, bovine *longissimus* muscles from 4 heifers (age <30 months) and 4 cows (age 3-5 years) were purchased from a local processor to represent two different physiological maturity groups. Muscles were stored at 4 ºC until the 7th day post-mortem. The dorsal epimysium was excised on the 7th day post-mortem with about 1 cm thick layer of meat attached, vacuum packaged and stored until use at –20 ºC. The heating experiments were carried out in 2 phases, presented as part A and B. The 'part A' experiments used epimysium from right side muscles and the part B experiments used epimysium from left side muscles. After thawing epimysium at 4 ºC, visible fat and muscle fibres were removed with a scalpel blade and two adjacent strips (2 cm wide) were cut along the length of epimysium. Subsequently, each strip was further cut into 2x2½ cm pieces. The weight, length and thickness (Dial Thickness Gauge No: 7301, Mitutoyo Corporation, Japan) were first recorded. The samples were placed in 50 mL polycarbonate tubes and 10 mL of de-ionized water (pH~7.5) was added. All epimysium samples prepared from a strip were given the same temperature treatment. 'Part A' experiments included heating at 55±2 ºC and 70±2 ºC. 'Part B' experiments included heating at 80±2 ºC and 95±2 ºC. Heating times (durations) within a temperature treatment were allocated to epimysium samples

in a random manner. At 55 ºC, epimysium samples were heated for 15, 30, 45, 60, 180, 540 and 1440 min; at 70 ºC, samples were heated for 5, 10, 15, 45, 60, 180 and 360 min. Similarly, at 80 and 95±2 °C epimysium samples were heated for 5, 10, 15, 45 and 60 min. Raw epimysium pieces cut from the same strips were immediately stored at -20 ºC to determine raw shear stress. The same treatment combination was given to two adjacent pieces of epimysium cut from a strip and those were considered as sub-samples. Each treatment combination was replicated with 4 different animals.

At the end of heating the aqueous phase was drained into 30 mL polycarbonate tubes, cooled to room temperature and then centrifuged at 31,000x*g* (J2-HC centrifuge, JA-17 rotor, Beckman Instruments, Palo Alto, CA, USA) for 30 min and at 20 ºC. The volume of the supernatant was adjusted to 10 mL with de-ionized water and stored at -20 ºC for the determination of thermolabile protein. Heated epimysium samples were stored at 4 ºC to determine the weight gain, length change and shear stress the following day. The raw epimysium remaining after sampling was diced, vacuum packed and stored at -20 ºC.

4.3.2 Proximate analysis

The frozen epimysium was first cut into small pieces and then repeatedly ground (Waring commercial blender, Waring Products, Torrington, USA) in liquid N_2 until no further reduction in connective tissue fragments could be attained. This tissue was used for both proximate and total collagen analysis.

Total moisture (AOAC 950.46 B, 1990), crude protein (AOAC 981.10, 1990) and crude fat (AOAC 960.39, 1990) contents of ground epimysium were determined in duplicate for the characterization of the raw epimysium from each animal.

4.3.3 Scanning electron microscopy (SEM)

Specimens for SEM were prepared on the 7th day post-mortem. About 75 cm^2 pieces were cut from the posterior end of the epimysium from one cow and heifer. After cleaning visible fat and muscle fibres, epimysium pieces were further diced into 5 pieces for random treatment allocation. One, randomly selected piece was used as the control. The latter was further diced into about 4 mm^2 pieces and immediately preserved in a buffer containing 3% gluteraldehyde and 0.1 M sodium cacodylate at 4 ºC, according to Olkowski et al. (2001) with modifications as described below. The other four pieces of epimysial tissue were heated at 55 ºC for 15 and 60 min and also at 70 ºC for 5 and 15 min in 10 mL of de-ionized water. After heat treatments, epimysium pieces were diced and preserved in buffer as described for the control. Later, epimysium pieces were washed in de-ionized water and dehydrated with an ascending series of ethanol and absolute acetone. Samples were lyophilized, fractured as desired with a scalpel and then stored in a desiccator until sputter-coated; first with carbon and then with gold. Samples were examined under a 15 kV electron beam of JEOL JSM-840A Scanning Electron Microscope.

4.3.4 Total collagen

Total collagen content of raw ground epimysium was determined according to the AOAC 990.26 (1990) procedure for hydroxyproline determination in meat and meat products. Briefly, about 0.4 g of epimysium was digested overnight with 3.5 M H_2SO_4 in an oven at 105±1 ºC. The hot digest was diluted to 100 mL with de-ionized water. After filtering an aliquot was stored at -20 ºC. Before chemical analysis, liquid samples were thawed at room temperature and appropriate dilutions were prepared. A sample aliquot of 2 mL was mixed with 1 mL of 1.41% chloramine T (w/v) and incubated for 20 min at room temperature. Then it was mixed with 1 mL of colour reagent (10 g 4-dimethylaminobenzaldehyde, 35 mL of 60% perchloric acid (w/w) and 65 mL of 2-propanol) heated at 60±1 ºC for 15 min. At the end of heating samples were

cooled to room temperature in a water bath. Absorbance was measured at 558 nm using Genesys 5 UV-VIS spectrophotometer (Milton & Roy Spectronic, Ivyland, PA, USA). Calibration standards were prepared using trans-4-hydroxy-L-proline (Sigma-Aldrich, St Louis, MO, USA). A conversion factor of 8 was used, as prescribed by AOAC 990.26 (1990), to convert hydroxyproline to collagen.

4.3.5 Hydrolysis with pronase

With some modifications, the procedure developed by Snowden & Weidemann (1978) was adopted for extracting amorphous collagen from the heat-treated epimysium. In brief, after shear force determination, epimysium was finely minced. About 0.8 g of that was placed in 10 mL polycarbonate centrifuge bottles (Beckman Instruments, Palo Alto, CA, USA) and mixed with 8 mL of a buffer containing 0.5 mg/mL pronase (protease from *Streptomyces griseus,* Sigma-Aldrich, St Louis, MO, USA) with the activity level of 5.2 units/mg, in 0.12 M Tris / 0.1 M $CaCl_2$ at pH 7.4 (Scott et al., 1981). Samples were hydrolysed overnight at room temperature on a shaker. At the end of hydrolysis, samples were centrifuged (Beckman Coulter Optima LE-80K ultracentrifuge, Beckman Instruments, Palo Alto, USA) for 30 min at 80,000x*g* and at 20 ºC. The volume of the supernatant was adjusted to 10 mL with de-ionized water and stored at -20 ºC for the subsequent determination of protein liberated by pronase and Ehrlich Chromogen (EC) contents. The pellet was discarded. The contribution of the enzyme pronase to protein data was regarded as negligible as 8 mL contained 4 mg of protein from this source.

4.3.6 Determination of thermolabile protein

Thermolabile and pronase liberated protein contents were determined according to the bicinchoninic acid (BCA) protein assay (Smith et al., 1985). Assay reagents A (sodium carbonate, sodium bicarbonate, sodium tartrate and BCA detection reagent in 0.1 N sodium hydroxide) and B (4% cupric sulphate pentahydrate) were mixed in the ratio 100:2 (v/v),

respectively (Assay reagents were purchased from Thermo Fisher Scientific Inc. Rockford, IL, USA). After appropriate dilution of test solutions, an aliquot of 100 µL was pipetted out and mixed with 2 mL of the reagent mixture in 15 mL glass tubes. After immediate vortexing for 30 s, tubes were heated at 60 ºC for 30 min in a pre-heated water bath. Contents in tubes were cooled to room temperature in another water bath before measuring absorbance at 562 nm (Genesys 5, UV-VIS spectrophotometer, Milton & Roy Spectronic, Ivyland, PA, USA). As described by Smith et al. (1985), gelatine had a lower absorbance than BSA at the same concentration and also the absorbance curve of gelatine at 562 nm was more curvilinear. Therefore, to improve accuracy in measuring absorbance of gelatine solutions, calibration standards were prepared from bovine skin gelatine type B (Sigma-Aldrich, St Louis, MO, USA) replacing traditionally used BSA.

4.3.7 Electrophoresis

The aqueous phase obtained after heating the epimysium and preserved at -20 °C was thawed to room temperature. Based on protein data from the BCA assay, calculated volumes of the aqueous phase from selected treatments were lyophilized in 1 mL micro centrifuge tubes to obtain 50 µg of dry protein. Then the whole lyophilized sample was mixed with 100 µL of 2X sample buffer (0.1 M Tris at pH 6.8, 2% sodium dodecyl sulphate, 20% glycerol, 0.001% bromophenol blue and 38% de-ionized water) containing 2-mercaptoethanol in 19:1 ratio, (v/v) respectively. The sample mixture was then heated to 95 ºC for 5 min. Resolution was carried out on polyacrylamide gels consisted of 8% resolution phase and a 4% stacking phase and each well was loaded with 3 µg of protein. Electrophoresis was carried out at a voltage of 176 V, in Tris (3.025 g/L) and glycine (14.415 g/L) buffer at pH 8.2, made in double de-ionized water. Gels were stained (0.01% coomasie brilliant blue, 50% methanol and 7% acetic acid) for 5 min and then de-stained overnight (10% acetic acid, 7.5% methanol). A pre-stained, broad range

molecular weight standard (Bio-Rad Laboratories, Hercules, CA, USA) was used as a molecular weight marker.

4.3.8 Determination of Ehrlich chromogen (EC)

EC content of the amorphous collagen released after pronase hydrolysis was determined according to Scott et al. (1981) as a measure of pyrrolic cross-links in epimysium. Sample and reagent solution (5% dimethyl amino benzaldehyde and 0.01% mercuric chloride in 4 N perchloric acid) were mixed in a 5.25: 1 (v/v) ratio, respectively in 2 mL micro tubes. The mixture was centrifuged immediately at 14,638 x *g* (Eppendorf Centrifuge 5415 C, Brinkmann Instruments Inc, Westbury, NY, USA) for 30 s at room temperature. Four minutes after mixing the sample and reagent, absorbance was measured at 572 nm (Genesys 5 UV-VIS spectrophotometer, Milton & Roy Spectronic, Ivyland, PA, USA). EC content was expressed as absorbance per gram of pronase liberated protein.

4.3.9 Determination of pyridinoline cross-links

Pyridinoline cross-link contents, a mix of pyridinoline and deoxypyridinoline, of heifer and cow epimysium, before and after heat treatment were determined using a Metra PYD enzyme linked immuno assay kit (Quidel Corporation, San Diego, CA, USA). This method was previously employed to determine pyridinoline cross-links in meat (Boutten et al., 2000; Listrat et al., 2007). Briefly, about 1 g of finely ground epimysium from 2 heifers and 2 cows were weighed into polypropyline tubes and 10 mL of de-ionized water added. Samples were heated to 70 ºC, and then maintained at the same temperature for 3 h. At the end of heating samples were centrifuged at 2,060x*g* for 15 min at 20 ºC and the aqueous phase was decanted. The residue was transferred into 50 mL Pyrex tubes with 20 mL of 6 M HCl. The contents were digested overnight at 110 ºC. Hydrolysate, after cooling to room temperature, was centrifuged at 31,000x*g* (Beckman Instruments, Palo Alto, CA, USA) for 10 min at 20 ºC. A volume of 300

µL of the supernatant was mixed with 300 µL of 6 M NaOH and 300 µL of 1 M Tris in a 50 mL beaker. Adding 6 M HCl or 6 M NaOH brought the final pH between 7 and 8. After appropriate dilutions, the above prepared sample solution was analysed for total pyridinoline and deoxypyridinoline contents, according to the method recommended by the supplier of the assay kit. Cross-link contents of raw epimysium also was determined using the same procedure, but without the heat treatment.

4.3.10 Determination of shear stress

Raw epimysium samples were thawed to 4 ºC. From each raw and cooked epimysium sample, three strips of 0.5 cm width were cut parallel to the visible fibre direction. The lengths of strips were not controlled as epimysium pieces shrank after heating. Strips at room temperature were sheared perpendicular to the fibre direction using TMS-Pro texture system (Food Technology Corp., USA) equipped with a Warner-Bratzler shear attachment and a 1000 N load cell. Maximum shear stress generated was expressed as the force applied on a unit cross sectional area of epimysium sample and also corrected for raw thickness (N/mm^2).

4.3.11 Statistical analysis

The treatments were allocated in a factorial arrangement and data were analyzed as a randomized complete block design by analysis of variance (ANOVA) using SAS-GLM procedure for RCBD of SAS 9.1 programme (SAS Institute Inc, USA) with a pre-determined significance level of $P<0.05$. The experimental model was separately applied to each temperature treatment because heating intervals used were different. An epimysium piece was the experimental unit. The animal was considered nested within maturity. The model included main effects of maturity and heating time and their two-way interactions. When interactions were significant, simple effects were generated as one-way ANOVA, where effect of heating

time was studied within each age group separately. Mean separations were performed using Least Significant Differences (LSD) of SAS programme.

4.4. Results of part A experiments

Epimysium was characterized based on protein, fat, moisture and collagen contents (Table 4.4.1). Accordingly, epimysium from heifers had about 38% protein on a raw weight basis and collagen accounted for about 89% of proteins present. Epimysium from cows had more proteins (44%) and more collagen on protein basis (91%) than heifers. Heifer epimysium had more fat (about 16%) than cow epimysium (about 9%). Cow and heifer epimysium were of similar moisture content. Cow epimysium had higher shear force values than epimysium from heifers.

Table 4.4.1 Characterization of epimysium from heifers and cows

	Heifer	Cow	P	LSD
Protein (g/100g raw epimysium)	38.4±1.7	43.5± 4.4	0.016	3.22
Fat (g/100g raw epimysium)	16.4±2.5	9.3 ± 4.0	0.027	3.95
Moisture (g/100g raw epimysium)	47.2±1.7	47.4±3.4	0.831	-
Collagen (g/100g raw epimysium)	36.4 ±2.9	40.1±4.2	0.114	3.77
Collagen (g/100 g Protein)	88.5±8.0	91.4±9.0	0.008	6.13
Shear stress (N/mm^2)	32.0±3.2	38.6±7.8	0.021	5.29

Mean ± SD are presented. N=4.

4.4.1 Weight gain

Weight gain was computed as *[(Wf-Wo) / Wo]*100* where, *Wo* and *Wf* are epimysium raw weight and the weight after heating, respectively. Epimysium gained weight by absorbing water but at the same time liberating proteins. Both at 55 and 70 °C, epimysial weight gain was not influenced by the maturity of the animal and this may be resulting from the insufficient age gap between heifers (<30 months) and cows (3-5 years) to demonstrate differences in properties. Heating time had significantly increased ($P<0.0001$) the epimysial weight gain (Table 4.4.2 and Table 4.4.3). At 55 °C, heating time had no effect on weight gain (water uptake) until

Table 4.4.2 Probabilities of main effects and their two-way interactions of physicochemical properties of cow and heifer epimysium at 55 ºC

	df	Weight gain (%)	Thickness change (%)	Thermolabile protein (g/100 g epimysium)	Pronase liberated protein (g/100g raw weight)	Sum of thermolabile & pronase released protein /Kjeldhal protein (w/w %)	EC/ g Pronase liberated protein	Shear stress (N/mm^2)
Maturity	1	P<0.7453	P<0.9225	P<0.0521	P<0.6804	P<0.1292	P<0.2466	P<0.0082
Maturity(block)	6	P<0.0001	P<0.0001	P<0.2163	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Time	6	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.1213	P<0.0001
Maturity*Time	6	P<0.0598	P<0.7617	P<0.0037	P<0.8588	P<0.0032	P<0.2583	P<0.4249

P- probability. df-degrees of freedom.

Table 4.4.3 Probabilities of main effects and their two-way interactions on physicochemical properties of cow and heifer epimysium at 70º C

	df	Weight gain (%)	Thickness change (%)	Thermolabile protein (g/100 g epimysium)	Pronase liberated protein (g/100g raw weight)	Sum of thermolabile & pronase released protein /Kjeldhal protein (w/w %)	EC/ g Pronase liberated protein	Shear stress (N/mm^2)
Maturity	1	P<0.3121	P<0.0180	P<0.0490	P<0.6909	P<0.3257	P<0.6867	P<0.0080
Maturity(block)	6	P<0.0001	P<0.0006	P<0.0554	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Time	6	P<0.0001	P<0.0006	P<0.0002	P<0.3839	P<0.0333	P<0.3059	P<0.0019
Maturity*Time	6	P<0.3580	P<0.5664	P<0.0044	P<0.4089	P<0.1749	P<0.6518	P<0.0571

P- probability. df-degrees of freedom.

up to 60 min, but further heating to 180, 540 and 1440 min had increased weight gain significantly (Table 4.4.4). A clear difference in weight gain between two adjacent heating times, at 70 °C, was observed only beyond 60 min heating (Table 4.4.5). Visual observation of data from the temperature treatments shows that temperature increase from 55 to 70 °C had a positive effect on epimysial weight gain. After 360 min heating at 70 °C weight of cow and heifer epimysium were 78 and 83% above raw weight, respectively. However, epimysial weight gain was not influenced by the maturity of animals after heating at 55 and 70 °C (Table 4.4.4). Comparison of epimysial weight gain (water uptake) data from two temperature treatments revealed that water uptake was faster at 70 °C than at 55 °C, i.e. for about 30% increase in weight (based on mean values) was reached within 5 min heating at 70 °C and after 180 min heating at 55 °C.

4.4.2 Thickness increase

Epimysium thickness increase was measured as *[(Tf-To) / To*100]* where *To* and *Tf* are epimysium thickness values before and after heating, respectively. The epimysium thickness increase at 55 ºC was not influenced by the maturity of the animal (Table 4.4.2 and Table 4.4.4) but at 70 ºC, thickness of cow epimysium increased more than that of heifer epimysium ($P<0.001$) (Table 4.4.3 and Table 4.4.5). Thickness of epimysium was significantly increased with increasing heating times at 55 ºC ($P<0.0001$) (Table 4.4.2 and Table 4.4.4) and at 70 ºC ($P<0.001$) (Table 4.4.3 and Table 4.4.5). At 55 ºC, a significant change in thickness values for two adjacent heating times was evident only after 540 min heating (Table 4.4.4). Similarly, heating over 180 min at 70 ºC had significantly increased the epimysial thickness values of two neighbouring heating times (Table 4.4.5). Apparently, temperature increase from 55 to 70 ºC had a positive effect on epimysial thickness increase of both cows and heifers. Obviously, thickness increase was faster at 70 ºC than at 55 ºC; thickness was increased by about 100%

Table 4.4.4 Effects of animal maturity and heating time at 55 ºC on physicochemical properties of epimysium.

		Physicochemical property						
		Weight gain (%)	Thickness change (%)	*Thermolabile protein (g/100 g epimysium)	Pronase liberated protein (g/100g raw weight)	*Sum of thermolabile & pronase released protein /Kjeldhal protein (w/w %)	EC*/g Pronase liberated protein	Shear stress (N/mm^2)
Maturity	Heifer	25.9±22.0	37.0±27.5	3.3±6.2	6.2±2.4	24.8±20.5	0.014±0.006	14.0±9.1
	Cow	23.2±19.5	38.1±37.3	0.84±0.7	5.3±3.7	14.0±9.0	0.018±0.004	28.9±9.2
	P	0.745 (NS)	0.923 (NS)	0.052 (NS)	0.052 (NS)	0.129	0.246 (NS)	0.008
	LSD	-	-	-	-	-	-	2.8
Heating time	15 min	9.3±3.6	16.2±8.0	0.27±0.2	4.2±1.3	10.9±3.8	0.013±0.004	32.3±6.7
	30 min	13.0±8.3	20.2±14.6	0.47±0.3	4.4±2.0	12.0±5.2	0.017±0.008	24.6±9.2
	45 min	13.5±8.3	24.7±15.2	0.50±0.3	4.7±2.2	12.7±5.5	0.016±0.005	24.6±10.4
	60 min	15.8±9.1	27.5±17.6	0.61±0.3	5.1±2.7	13.9±6.2	0.014±0.002	21.4±8.1
	180 min	26.3±14.0	38.8±24.2	1.3±0.8	6.3±3.4	18.8±9.7	0.016±0.005	19.2±10.9
	540 min	41.1±18.7	50.2±29.8	3.4±2.5	7.5±3.4	27.3±13.9	0.016±0.004	16.7±12.8
	1440 min	52.7±26.4	74.7±49.1	8.0±6.1	8.2±4.2	40.5±30.9	0.019±0.007	11.3±10.0
	P	0.0001	0.0001	0.0001	0.0001	0.0001	0.121 (NS)	0.0001
	LSD	10.1	21.0	3.31	1.3	8.93	-	5.3

The means±SD of physicochemical properties with significant main effects are listed above. * Symbol indicates properties with significant interactions.
NS= Not Significant. EC*- EC measured as absorbance at 572 nm.

Table 4.4.5 Effects of animal maturity and heating time at 70 ºC on physicochemical properties of epimysium

		Physicochemical property						
		Weight gain (%)	Thickness change (%)	*Thermolabile protein (g/100 g epimysium)	Pronase liberated protein (g/100g raw weight)	Sum of thermolabile & pronase released protein /Kjeldhal protein (w/w %)	[1]EC/g Pronase liberated protein	Shear stress (N/mm^2)
Maturity	Heifer	56.2±21.7	84.5±29.2	3.4±5.1	14.2±4.0	43.0±16.0	0.017±0.003	2.1±0.7
	Cow	45.0±25.4	148.4±56.3	0.85±0.7	13.1±4.2	34.6±9.0	0.019±0.005	11.6±5.9
	P	0.312 (NS)	0.018	0.049	0.699 (NS)	0.326 (NS)	0.686 (NS)	0.008
	LSD	-	17.5	1.4	-	-	-	1.3
Heating time	5 min	31.8±14.6	94.1±55.6	0.53±0.4	13.9±4.2	35.7±10.5	0.018±0.003	8.6±7.5
	10 min	37.1±14.9	96.8±30.3	0.82±0.5	12.7±6.6	32.7±15.9	0.017±0.005	6.8±5.2
	15 min	36.7±16.6	109.5±64.6	0.85±0.6	14.2±4.0	36.9±14.3	0.017±0.005	7.2±6.4
	45 min	45.6±16.5	92.4±45.9	1.4±0.9	14.2±3.8	38.4±10.4	0.017±0.005	9.1±7.5
	60 min	54.3±14.0	119.2±54.3	1.5±1.0	14.9±3.6	40.5±9.7	0.018±0.004	7.3±4.7
	180 min	68.5±20.9	145.2±53.2	3.0±2.4	13.6±3.6	40.9±10.9	0.019±0.006	5.1±3.8
	360 min	80.5±25.7	158.3±53.9	6.9±8.5	12.0±3.0	46.9±22.2	0.019±0.005	3.9±2.4
	P	0.0001	0.001	0.001	0.383 (NS)	0.033	0.305 (NS)	0.002
	LSD	11.5	32.8	2.6	-	8.1	-	2.5

The means±SD of main effects are listed above. * Symbol indicates properties with significant interactions.
NS= Not Significant. [1]EC-EC measured as absorbance at 572 nm.

after 10 min heating at 70 ºC, which is a value not reached within 24 h heating of epimysium at 55 ºC.

4.4.3 Thermolabile protein and pyridinoline cross-links

Release of thermolabile protein from the epimysium was significantly influenced by the interaction between maturity*heating time at both 55 ($P<0.05$) (Table 4.4.2) and at 70 ºC ($P<0.05$) (Table 4.4.3). Heating time had a significant effect on the release of thermolabile proteins at 55 ºC ($P<0.0001$) and at 70 ºC ($P<0.001$). According to the 2-way interaction means at 55 ºC, the amount of protein released by heifer epimysium after 540 and 1440 min heating was significantly higher than protein released at all other treatments for heifer and cow epimysium (Table 4.4.6). After 1440 min heating at 55 ºC, the amount of protein released from heifer epimysium was 7 times more than that of cow epimysium, indicating the susceptibility differences between cow and heifer epimysium to aqueous heating. Heifer epimysium had released significantly higher amounts of protein after 180 and 360 min heating at 70 ºC than all other treatments for heifer and cow epimysium (Table 4.4.7). For example, after 360 min heating at 70 ºC heifer and cow epimysium had liberated 1.8±1.2% and 11.9±10% proteins respectively, on weight basis. Maturity of animals had shown a tendancy to have a significant effect on thermolabile protein release at the two temperatures investigated ($P=0.05$); heifers had released more thermolabile protein than cows (Table 4.4.4 and Table 4.4.5).

During heating in water at 55 ºC, epimysium from cows (Figure 4.1) and heifers (Figure 4.2) had liberated single strands (α1 and α2 chains, >116 kDa) and double strands (2α chains, >200 kDa) of collagen with other unidentified connective tissue proteins. Molecular weight of an α chain of collagen is about 100 kDa and that o 2α chains is about 200 kDa (Gómez-Guillén et al, 2002; Dreisewerd et al., 2004). Heifer epimysium released single and double strands of

Table 4.4.6 Two-way interaction means for physicochemical properties of epimysium at 55 ºC

	Interaction		Interaction means
Thermolabile protein	Maturity	Heating time (min)	
(g/100 g epimysium)	Heifer	15	0.23±0.1
		30	0.42±0.1
		45	0.58±0.3
		60	0.64±0.3
		180	1.80±0.9
		540	5.60±1.6
		1440	14.0±12.0
	Cow	15	0.31±0.2
		30	0.53±0.4
		45	0.43±0.3
		60	0.58±0.4
		180	0.84±0.4
		540	1.20±0.4
		1440	2.00±1.1
Sum of thermolabile &	Heifer	15	12.4±4.2
pronase released protein/		30	13.9±5.1
Kjeldahl protein (w/w %)		45	14.7±5.2
		60	15.1±4.0
		180	22.2±9.7
		540	37.1±10.5
		1440	58.5±33.9
	Cow	15	9.5±3.3
		30	10.1±5.4
		45	10.6±5.7
		60	12.6±8.4
		180	15.3±9.8
		540	17.6±9.5
		1440	22.5±14.8

The means±SD of physicochemical properties for which Maturity*Heating time interaction was significant are presented.

Table 4.4.7 Two-way interaction means for thermolabile protein of epimysium released at 70 ºC

	Interaction		Interaction means
Thermolabile protein	Maturity	Heating time (min)	
(g/100 g epimysium)	Heifer	5	0.6±0.1
		10	1.0±0.5
		15	1.2±0.7
		45	1.9±0.9
		60	2.3±0.9
		180	5.0±1.6
		360	11.9±10.1
	Cow	5	0.50±0.6
		10	0.65±0.6
		15	0.48±0.40
		45	0.75±0.5
		60	0.78±0.5
		180	1.00±0.8
		360	1.8±1.2

The means±SD of physicochemical properties for whch Maturity*Heating time interaction was significant are presented.

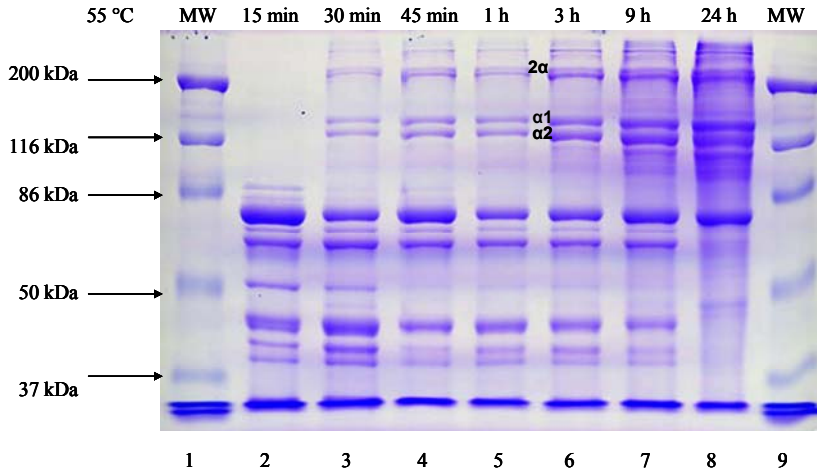


Figure 4.1 SDS gels showing protein bands liberated from cow epimysium at 55 °C. Resolution carried out on 8% polyacrylamide gels with a 4% stacking gel. Three µg of protein was applied to each well. As heating time increased from lane 2-8 band thickness of single (α1, α2) and double strands (2α) increased. MW stands for molecular weight standard.

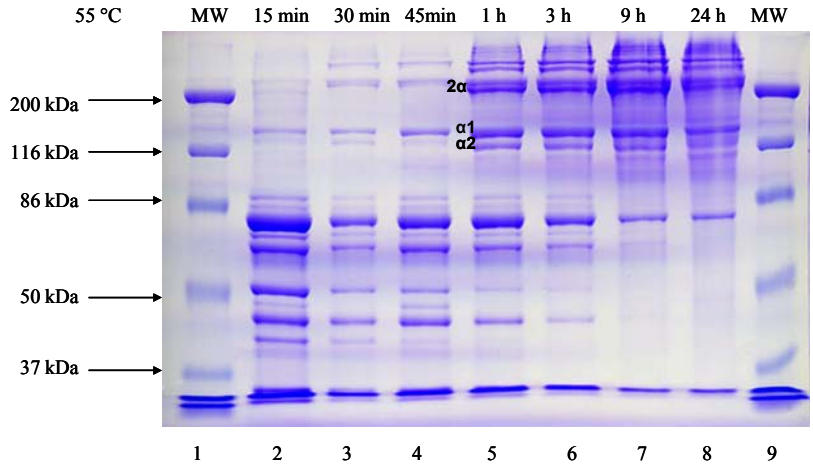


Figure 4.2 SDS gels showing protein bands liberated from heifer epimysium at 55 °C. Resolution carried out on 8% polyacrylamide gels with a 4% stacking gel. Three µg of protein was applied to each well. As heating time increased from lane 2-8 band thickness of single (α1, α2) and double strands (2α) increased. MW stands for molecular weight standard.

collagen starting from 15 min heating at 55 ºC but cow epimysium started releasing them only after 30 min heating at 55 ºC. At corresponding heating times, apparently heifer epimysium released more double strands than cow epimysium. Molecular weights of protein bands produced at 70 ºC were similar to those observed at 55 ºC for both cow and heifer epimysium, however band thickness increased at 70 ºC indicating liberation of more proteins than at 55 ºC (data not shown).

To understand the effect of aqueous heating on the release and retention of pyridinoline cross-links in cow and heifer epimysium, an immuno assay of pyridinoline cross-links was carried out with two animals per maturity group (Table 4.4.8). Clearly, cow epimysium contains more pyridinoline cross-links (per gram of protein) than heifer epimysium, before treatments. After heating for 3 h at 70 ºC, cow and heifer epimysium had retained about 1/3 of the original cross-link content. After correcting cross-link contents to the amounts of protein retained in heated epimysium, it was noted that, at the end of heating cow epimysium retained more cross-links in the residue than heifer epimysium.

Table 4.4.8 Pyridinoline and deoxypyridinoline cross-link contents of epimysium before heating and retained in epimysium after heating at 70 ºC

	Pyridinoline and deoxypyridinoline cross-links (n moles/g of crude protein)	
	Heifer	Cow
Raw epimysium	63.9±13.1	167.3±22.3
Epimysium heated at 70 ºC for 3 h	16.5±2.0	56.2±15.3

4.4.4 Pronase liberated protein

According to Snowden and Weidemann (1978), some of the molecules in a collagen fibre become amorphous during heating and remained attached to the matrix. Those amorphous areas

were released by the action of pronase. Differences between cow and heifer epimysium (maturity) did not influence the amount of amorphous collagen produced at either 55 or 70 ºC (Table 4.4.2 and Table 4.4.3). At 55 ºC, gradually increasing heating times in water had significantly increased the amount of amorphous collagen produced ($P<0.0001$) (Table 4.4.4). Similar amounts of amorphous collagen were produced between 15 to 45 min heating at 55 ºC. Heating times over 60 min at 55 ºC had produced slightly higher amounts of amorphous collagen than the shorter heating times. The highest amount of amorphous collagen (8.2±4.4%) was produced after 24 h heating at 55 ºC (Table 4.4.4). At 70 ºC, neither animal maturity nor heating time had any effect on the amount of amorphous collagen extracted from epimysium with pronase (Table 4.4.3 and Table 4.4.5). In other words, amounts of amorphous collagen extracted from cow and heifer epimysium were similar after all heat treatments. For example, at 70 ºC this is about 14% of the raw weight of epimysium (Table 4.4.5). Thus, the temperature increase from 55 to 70 ºC had remarkably reduced the time requirement for collagen to become amorphous.

The total amount of protein liberated from epimysium after combined treatments of heating and pronase hydrolysis was expressed as a percentage of total epimysial protein (on a weight basis), *[(Thermolabile+Pronase liberated protein/Total Kjeldahl protein)*100]*. Heating time had a significant effect on the sum of all proteins released at 55 ºC ($P<0.0001$) and 70 ºC ($P<0.05$) (Table 4.4.2 and Table 4.4.3). After heating at 55 ºC, the sum of all proteins released from epimysium was influenced by the interaction, maturity*time ($P<0.05$) (Table 4.4.2). A significant increase in sum of proteins released was noted for both cow and heifer epimysium after 540 and 1440 min heating of epimysium at 55 ºC, however the increase observed for heifer epimysium was larger than the increase observed for cow epimysium (Table 4.4.6). This might have contributed to the observed interaction. Further, heifer epimysium had liberated more protein than cow epimysium at corresponding heating times; for example, after 1440 min

heating at 55 ºC, heifer and cow epimysium had released 58.5±34% and 22.5±15% of its protein (Table 4.4.6). This showed that proteins of heifer epimysium were more prone to thermal denaturation than that of cow epimysium.

Also, after a long exposure to a relatively low temperature (55 ºC), cow epimysium had retained more proteins (77.5%) than the heifer epimysium (41.5%). However, after heating at 70 ºC, the sum of all proteins released (on a protein basis) was gradually increased with the heating time and were in the range of 33-47% (Table 4.4.5). Animal maturity had no effect on total proteins at 70 ºC (Table 4.4.3).

4.4.5 Ehrlich chromogen (EC)

EC cross-links (pyrrolic cross-link) in amorphous areas of collagen were measured. EC content was reported as absorbance at 572 nm per unit weight (g) of pronase liberated protein from epimysium, due to non-availability of EC standards. EC released with the amorphous collagen was neither influenced by the maturity of animals nor by the heating times at both 55 (Table 4.4.2) and 70 ºC (Table 4.4.3). Apparently, temperature increase from 55 (Table 4.4.4) to 70 ºC (Table 4.4.5) also had no effect on the amount of 'EC/pronase liberated protein' released from cow and heifer epimysium. As the amount of EC present in a unit weight (g) of amorphous collagen was observed to be a constant it was assumed that EC cross-links were uniformly distributed in collagen that had become amorphous during heating. A preliminary study carried out on EC contents of thermolabile protein extracts from epimysial heating at 55 and 70 ºC had shown consistent but almost negligible absorbance values for all treatment combinations. Therefore, analysis of EC in thermolabile protein extracts was abandoned.

4.4.6 Shear stress

Shear force is a widely used mechanical measure to indicate tenderness/toughness of meat. This measure was adapted for use on epimysial connective tissues and corrected for differences in sectional area resulting in shear stress values. Shear stress values of raw epimysium from cows (~39.6 N/mm^2) were considerably higher than that from heifers (~30.8 N/mm^2) (Table 4.4.1). Maturity of animals had significantly influenced the shear stress values of epimysium after heating. Thus, epimysium from heifers had lower shear stress values than epimysium from cows at 55 ºC (Table 4.4.2 and Table 4.4.4) and also at 70 ºC ($P<0.05$) (Table 4.4.3 and Table 4.4.5). For example, reported shear stress value of cow epimysium at 70 °C was 11.6±6 N/mm^2 and that of heifer epimysium was 2.1±0.7 N/mm^2. Longer heating times also had significantly reduced the shear stress at 55 ºC ($P<0.0001$) and at 70 ºC ($P<0.05$) (Table 4.4.2 and Table 4.4.3). After 24 h heating at 55 °C, shear stress of heifer epimysium (about 1.8 N/mm^2) was about 10 times lower than that of cow epimysium (about 20.8 N/mm^2) (data not shown). The heat treatment at 70 °C had considerably reduced the shear stress values of epimysium (considering the means) compared to the corresponding values at 55 °C (Table 4.4.4 and Table 4.4.5). For example, 5 min exposure to 70 °C had reduced shear stress to about ~8.6 N/mm^2 (Table 4.4.5), which was a >3 fold reduction compared to that of raw heifer epimysium (~30.8 N/mm^2) and also a >4 fold reduction compared to shear stress of raw cow epimysium (~39.6 N/mm^2).

4.4.7 Correlations

Weight gain by heifer epimysium was positively correlated with thickness increase ($r=0.78$, $P<0.0001$) and thermolabile protein ($r=0.51$, $P<0.0001$) and negatively correlated with shear stress ($r=-0.74$, $P<0.0001$) (Table 4.4.9). Similar observations were made with cow epimysium where weight gain by cow epimysium was positively correlated with thickness increase ($r=0.81$, $P<0.0001$) and thermolabile protein ($r=0.64$, $P<0.0001$) and negatively correlated with shear

Table 4.4.9 Pearson correlations of physicochemical properties of heifer epimysium heated at 55 and 70 ºC[1]

	Heifer epimysium							
	Temperature	Time	Shear stress	Thermolabile protein	Thickness change	Weight gain	Pronase liberated protein	EC/Pronase liberated protein
Temperature	1							
Time	-0.314 P<0.843	1						
Shear stress	-0.683 P<0.0001	-0.257 P<0.056	1					
Thermolabile protein	0.009 P<0.945	0.648 P<0.0001	-0.339 P<0.011	1				
Thickness change	0.649 P<0.0001	0.255 P<0.058	-0.711 P<0.0001	0.633 P<0.0001	1			
Weight gain	0.578 P<0.0001	0.377 P<0.004	-0.764 P<0.0001	0.514 P<0.0001	0.781 P<0.0001	1		
Pronase liberated protein	0.714 P<0.0001	-0.248 P<0.066	-0.538 P<0.0001	-0.067 P<0.623	0.313 P<0.019	0.303 P<0.023	1	
EC/Pronase liberated protein	0.297 P<0.026	0.089 P<0.511	-0.232 P<0.086	-0.055 P<0.687	0.209 P<0.120	0.122 P<0.372	-0.052 P<0.700	1

N=56.

[1]-Correlations were studied combining the data from all heating times and temperatures to understand the universal effect of heating on epimysial properties

stress (r=-0.78, P<0.0001) (Table 4.4.10). Correlations computed on combined data from cows and heifers (Table 4.4.11) had shown weak relationships between parameters indicating weak universal relationships between parameters. For example, correlation between weight gain and thermolabile protein content (r=0.44, P<0.0001) was lower than those for cow and heifer epimysium.

Thickness increase of heifer epimysium had shown significant correlations with proteins liberated (r=0.63, P<0.0001) and shear stress (r=-0.71, P<0.0001) (Table 4.4.9). Similarly, thickness increase in cow epimysium had shown correlations with proteins liberated (r=0.43, P<0.0012) and shear stress (r=-0.89, P<0.0001) (Table 4.4.10). A weak correlation was observed between thermolabile protein and shear force of epimysium from heifers (r=-0.34; P<0.01) (Table 4.4.9) and cows (r=-0.39; P<0.003) (Table 4.4.10). Similarly, thermolabile protein of heifer epimysium had shown weak correlations with thickness change (r=0.63, P<0.0001) and weight gain (r=0.51, P<0.0001). Thermolabile proteins of cow epimysium also had shown weak correlations with thickness change (r=0.43, P<0.001) and weight gain (r=64, P<0.0001). According to pooled data, the correlation between thermolabile protein content and thickness change was further reduced (r=0.22, P<0.05) (Table 4.4.11) indicating these two parameters were poorly related in general.

Pronase liberated protein of heifer epimysium had shown correlations with weight gain (r=0.3034, P<0.05), thickness increase (r=0.31, P<0.05) and shear force (r=-0.54, P<0.0001) (Table 4.4.9). Similarly, pronase liberated protein of cow epimysium had shown correlations with weight gain (r=0.60, P<0.0001), thickness increase (r=0.85, P<0.0001) and shear force (r=-0.85, P<0.0001) (Table 4.4.10). In pooled data correlations between pronase liberated protein and shear force became lower than those observed for cow epimysium (r=-0.62, P<0.0001)

Table 4.4.10 Pearson correlations of physicochemical properties of cow epimysium heated at 55 and 70 ºC[1]

	Cow epimysium							
	Temperature	Time	Shear stress	Thermolabile protein	Thickness change	Weight gain	Pronase liberated protein	EC/Pronase liberated protein
Temperature	1							
Time	-0.337 P<0.015	1						
Shear stress	-0.747 P<0.0001	-0.015 P<0.911	1					
Thermolabile protein	-0.004 P<0.980	0.571 P<0.0001	-0.392 P<0.003	1				
Thickness change	0.758 P<0.0001	-0.057 P<0.678	-0.887 P<0.0001	0.427 0.001	1			
Weight gain	0.431 P<0.001	0.219 P<0.108	-0.777 P<0.0001	0.645 P<0.0001	0.809 P<0.0001	1		
Pronase liberated protein	0.791 P<0.0001	-0.197 P<0.150	-0.847 0.0001	0.229 P<0.093	0.849 P<0.0001	0.604 P<0.0001	1	
EC/Pronase liberated protein	-0.139 0.313	-0.073 P<0.595	0.115 P<0.405	-0.064 0.642	-0.142 P<0.302	-0.136 0.322	-0.121 P<0.380	1

N=56.

[1]-Correlations were studied combining the data from all heating times and temperatures to understand the universal effect of heating on epimysial properties

Table 4.4.11 Pearson correlations of combined data from cow and heifer epimysium[1]

	Cow epimysium							
	Temperature	Time	Shear stress	Thermolabile protein	Thickness change	Weight gain	Pronase liberated protein	EC/Pronase liberated protein
Temperature	1							
Time	-0.320 P<0.0006	1						
Shear stress	-0.606 P<0.0001	-0.091 P<0.430	1					
Thermolabile protein	0.003 P<0.972	0.481 P<0.0001	-0.346 P<0.0002	1				
Thickness change	0.660 P<0.0001	0.044 P<0.648	-0.546 P<0.0001	0.222 P<0.019	1			
Weight gain	0.502 P<0.0001	0.298 P<0.002	-0.697 P<0.002	0.442 P<0.0001	0.682 P<0.0001	1		
Pronase liberated protein	0.752 P<0.0001	-0.219 P<0.021	-0.625 P<0.0001	-0.018 P<0.849	0.641 P<0.0001	0.454 P<0.0001	1	
EC/Pronase liberated protein	-0.096 P<0.315	-0.051 P<0.593	0.124 P<0.196	-0.037 P<0.703	-0.096 0.316	-0.104 P<0.278	-0.092 P<0.339	1

N=112.

[1]-Correlations were studied excluding the effect of heating time and temperature

(Table 4.1.11). Thus, it is clear that correlations for measured parameters varied between maturity groups studied.

4.4.8 Scanning electron microscopy (SEM)

Scanning electron micrographs of raw epimysium, for cows and heifers, had shown that collagen fibres were mostly oriented parallel to each other as layers but an irregular distribution of fibres at other angles was common (Figure 4.3a). The organization of fibres from raw cow and heifer epimysium were similar, except that fibres in cow epimysium were more closely arranged in the space (Figure 4.4 a and b). After heating in water for 15 min at 55 °C, intermittent melting of collagen fibres was evident and the tissue had lost the fibrous structure (Figure 4.3 b). Complete melting of fibres was visible in exposed areas, after 1 h heating at 55 °C (Figure 4.3 c). Fibril structure of collagen was still visible at 70 °C, with some irregular melting of fibres after 5 min heating (Figure 4.3 d). A meshwork of fibres, resembling a basket weave, was exposed in some areas of the epimysium after 15 min heating at 70 °C (Figure 4.3e and Figure 4.3f). It was noted that heat induced melting of fibres showed a random distribution across the epimysium and unaffected fibres laid adjacent to melted fibres. Fibre melting was less common for cow epimysium (Figure 4.4c and Figure 4.4d) than heifer epimysium (Figure 4.3 b and Figure 4.3) after 15 and 60 min heating at 55 ºC. Evidently, heating to 70 °C had a clear effect on melting of fibres of cow epimysium (Figure 4.4e and Figure 4.4f). As observed from micrographs, fibres of heifer epimysium were more susceptible to heat denaturation at 55 °C (Figure 4.3c and Figure 4.4d) and 70 °C (Figure 4.3e and Figure 4.4f) than fibres from cow epimysium.

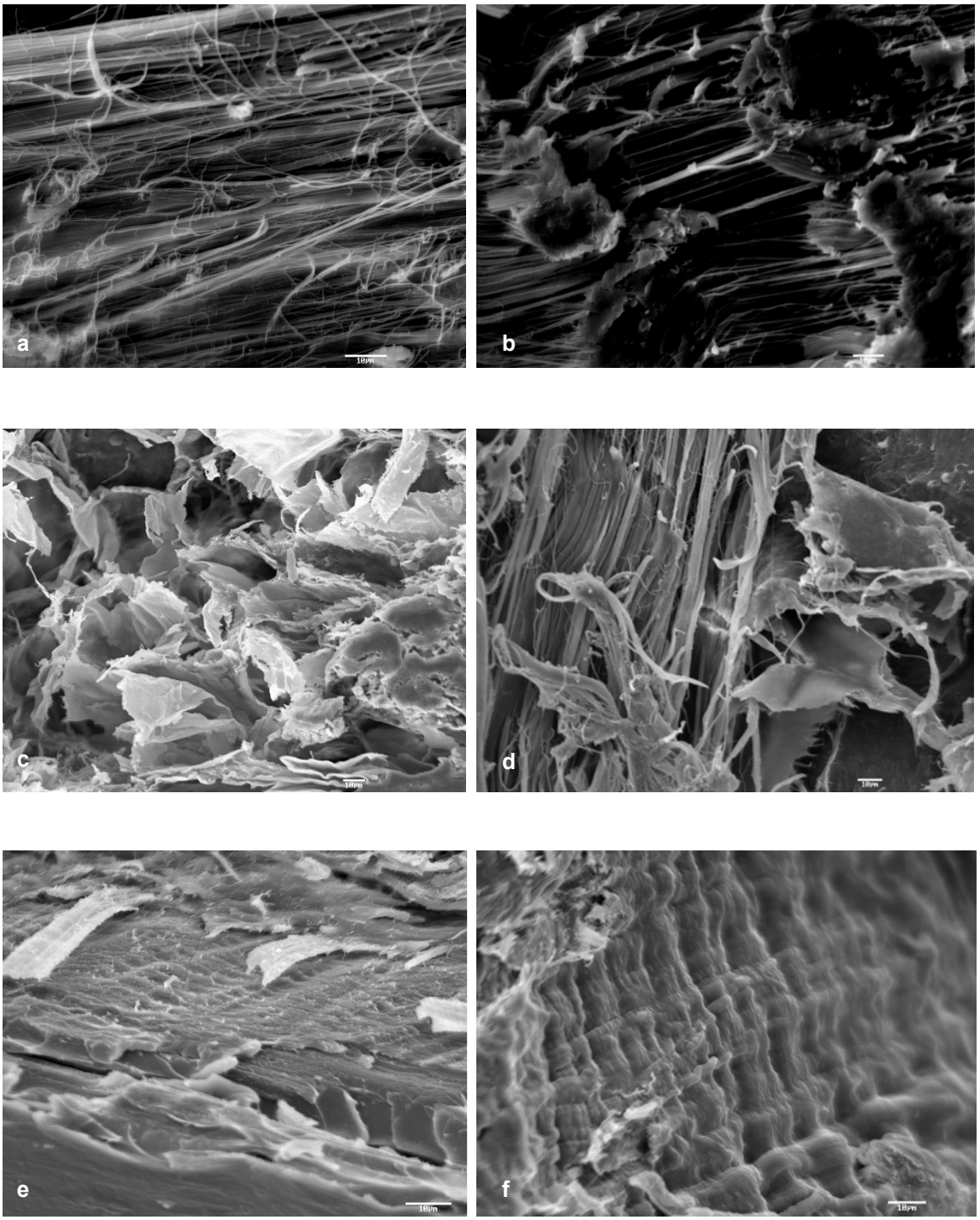


Figure 4.3 Scanning electron micrographs of heifer epimysium before and after heating: (a) Raw epimysium; (b) Epimysium after 15 min heating at 55 °C; (c) Epimysium after 1 h heating at 55 °C; (d) Epimysium after 5 min heating at 70 °C; (e) and (f) Epimysium after 15 min heating at 70 °C. Scale bar = 10 μm.

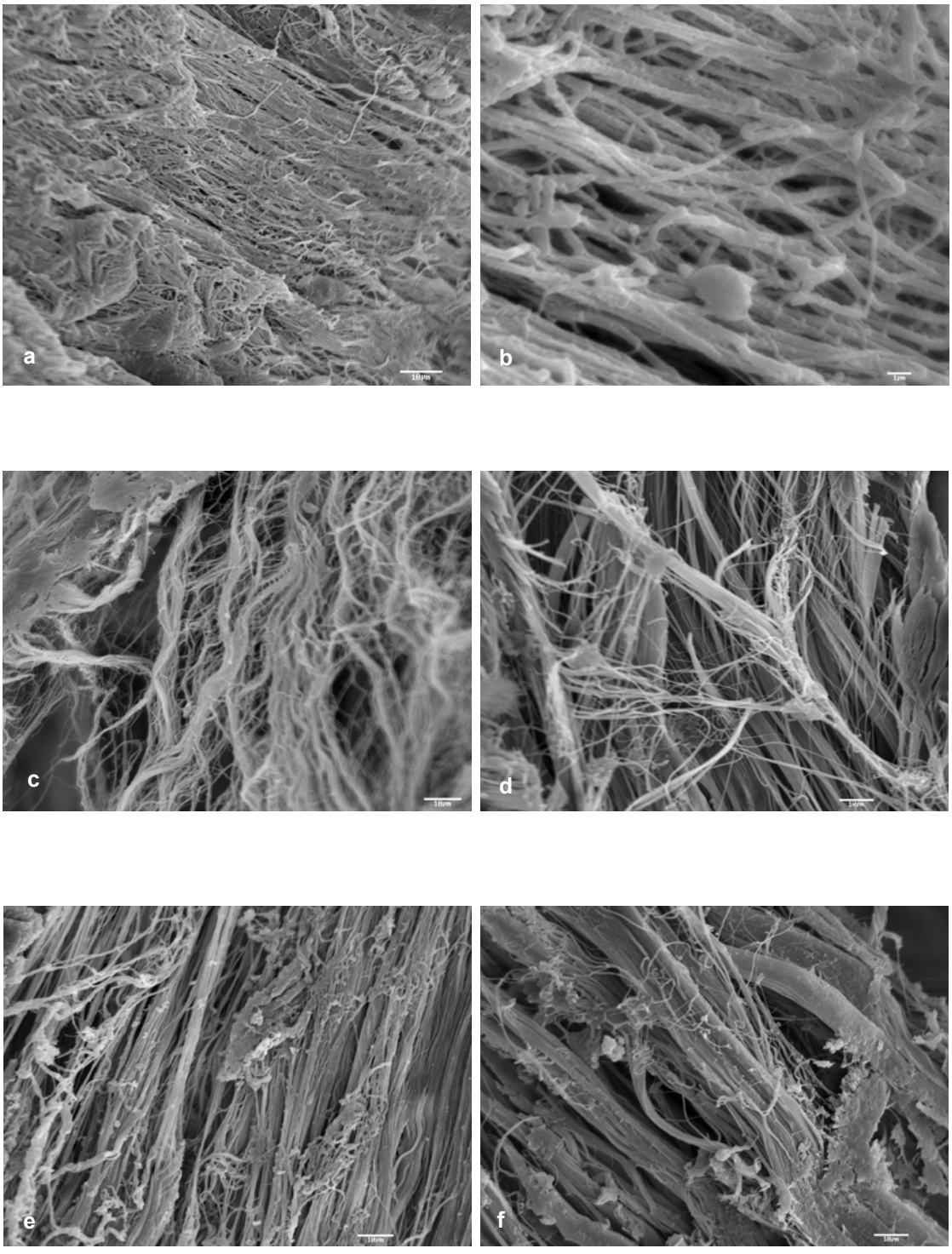


Figure 4.4 Scanning electron micrographs of cow epimysium before and after heating: (a) Raw epimysium; (b) Fibres of raw epimysium at x10 000; (c) Epimysium after 15 min heating at 55 °C; (d) Epimysium after 1 h heating at 55 °C; (e) Epimysium after 5 min heating at 70 °C; (f) Epimysium after 15 min heating at 70 °C. Scale bar of (b)=1 μm. Scale bar of other micrographs=10 μm).

4.5 Results of Part B experiments

According to the statistical model employed the two-way and three-way interactions among animal age, temperature and heating time did not have a significant effect on the following physicochemical parameters; thickness increase, shrinkage, shear force, thermolabile and pronase released proteins of epimysium (Table 4.5.1). Thus, the (main) effects of maturity, temperature and heating time were listed for those parameters. When interactions were significant for measured parameters, interaction means were reported.

4.5.1 Weight gain

During cooking epimysium released water-soluble constituents while absorbing water into the matrix and the net effect was designated as weight gain. Epimysial weight gain was significantly influenced by the heating time and the interaction, maturity*temperature ($P<0.05$) (Table 4.5.1). After heating for 45 min (~43% weight gain) and 60 min (~46% weight gain) either at 80 or 95 °C, epimysium had gained significantly higher weight than heating for short durations (LSD=6.4; $P<0.001$); values for weight gain after 5 and 10 min heating at 80 and 95 °C were ~33%. As temperature was increased from 80 to 95 °C, heifer epimysium had gained more weight but this was not observed for cow epimysium (Table 4.5.2). This might be the reason for the observed interaction. At 95 °C, cow epimysium had increased its weight by 38% and weight of heifer epimysium was increased by 47.5±8.6%.

4.5.2 Thickness increase

Thickness increase in epimysium was computed as $[(Tf - To) / To]*100$ where *To* was the raw thickness and *Tf* was the thickness after heating. At 80 and 95 °C, epimysial thickness change was not affected by maturity of animal, temperature or heating time (Table 4.5.1 and Table 4.5.3). However, a trend of cow epimysium gaining a higher thickness than heifer epimysium

Table 4.5.1 Probabilities of main effects and their two-way and three-way interactions of physicochemical properties of epimysium following heating at 80 and 95 °C

	Physicochemical property						
	Weight gain (%)	Thickness change (%)	Shrinkage (%)	Thermolabile protein (g/100g raw weight)	Pronase liberated protein (g/100g raw weight)	Sum of thermolabile & pronase released protein	Shear stress (N/mm^2)
Maturity	P<0.6630	P<0.0754	P<0.0016	P<0.8948	P<0.6883	P<0.1050	P<0.0083
Animal(Maturity)	P<0.0001	P<0.0001	P<0.0029	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Temperature	P<0.0017	P<0.3426	P<0.8121	P<0.0001	P<0.0001	P<0.0307	P<0.0001
Maturity*Temperature	P<0.0021	P<0.1196	P<0.1221	P<0.2515	P<0.1196	P<0.0180	P<0.7016
Time	P<0.0002	P<0.5753	P<0.9336	P<0.0001	P<0.0222	P<0.6243	P<0.0128
Maturity*Time	P<0.7224	P<0.6398	P<0.7636	P<0.4867	P<0.5887	P<0.5847	P<0.7743
Maturity*Temperature*Time	P<0.3995	P<0.9402	P<0.9502	P<0.6752	P<0.5236	P<0.5663	P<0.9999

Table 4.5.2 Two-way interaction means for physicochemical properties of epimysium at 80 and 95 ºC

	Interaction		Interaction means
Weight gain (%)	Maturity	Temperature (C º)	
	Heifer	80	32.6±12.4
		95	47.5±8.6
	Cow	80	38.0±15.2
		95	38.2±10.4
Sum of thermolabile & pronase released protein/ Kjeldahl protein (%)	Heifer	80	46.0±6.6
		95	42.3±7.4
	Cow	80	37.0±4.3
		95	37.2±3.8

The means±SD of physicochemical properties for which Maturity*Temperature interaction was significant are presented.

Table 4.5.3 Physicochemical properties of epimysium at 80 and 95 ºC as influenced by animal maturity, heating time and temperature

		Physicochemical property						
		*Weight gain (%)	Thickness change (%)	Shrinkage (%)	Thermolabile protein (g/100g raw weight)	Pronase liberated Protein (g/100g raw weight)	*Sum of thermolabile & pronase released protein/ Kjeldahl protein (%)	Shear stress (N/mm^2)
Maturity	Heifer	39.2±13.0	102.0±59.1	46.2±10.02	1.3±1.1	15.3±2.4	44.2±7.4	2.8±0.65
	Cow	38.4±12.5	209.6±140.6	68.3±6.08	1.7±1.4	14.7±2.0	37.1±4.0	6.5±2.2
	P	0.663 (NS)	0.075 (NS)	0.002	0.894 (NS)	0.688 (NS)	0.105 (NS)	0.008
	LSD	-	-	3.97	-	-	-	0.72
Temperature	80 °C	34.9±13.6	121.3±13.6	55.3±14.4	0.87±0.61	16.0±2.3	41.5±7.1	4.84±2.7
	95 °C	42.9±10.5	176.9±146.3	56.4±13.5	2.1±1.6	14.1±1.8	39.8±6.4	3.98±2.9
	P	0.002	0.342 (NS)	0.812 (NS)	0.0001	0.0001	0.031	0.0001
	LSD	4.02	-	-	0.31	0.54	1.60	0.71
Heating time	5 min	33.2±8.4	134.0±90.7	54.6±13.0	0.88±0.6	15.9±2.3	41.3±7.1	5.2±3.2
	10 min	33.1±10.4	151.6±108.7	56.9±13.2	1.1±0.9	15.2±2.0	39.6±6.7	4.9±3.1
	15 min	38.5±12.9	141.4±87.6	55.4±12.7	1.6±0.9	15.2±2.6	40.3±7.8	4.7±2.8
	45 min	43.4±13.7	153.3±128.2	55.7±15.6	1.9±1.5	14.6±2.4	40.6±7.0	3.6±2.2
	60 min	46.0±13.2	165.2±159.1	56.6±16.7	2.3±1.9	14.5±1.5	41.3±5.9	3.6±2.8
	P	0.001	0.575 (NS)	0.933 (NS)	0.0001	0.022	0.624 (NS)	0.012
	LSD	4.03	-	-	0.50	0.85	-	1.13

The means±SD of physicochemical properties with significant main effects are presented.
* Symbol indicates parameters with significant interactions.

during heating was noted (P=0.075) (Table 4.5.3). The high variability in thickness of cow epimysium samples might have contributed to the lack of a statistical difference between two temperature treatments.

4.5.3 Shrinkage

Shrinkage was computed as *[(Lo – Lf) / Lo)*100]*, where *Lo* is the initial length of epimysium piece, *Lf* is the length after heating. It was noted that length after heating was measured when samples reached 4 ºC. Collagen dominant epimysium shrank (decreased in length) considerably as a result of heating and that was significantly influenced by the maturity of animal (Table 4.5.1). After heating cow epimysium had shrank (68±6%) significantly more than heifer epimysium (46±10%) at corresponding treatments (Table 4.5.3). The length of the heating time (a maximum of 60 min) or temperature of heating (80 or 95 ºC) did not have any effect on epimysial shrinkage (Table 4.5.1).

4.5.4 Thermolabile protein release

Cow epimysium had significantly higher amounts of total protein, measured as Kjeldhal N, (43.5±4.4%) than heifer epimysium (38.4±1.3%) on a raw weight basis. About 90% of these epimysial proteins were determined to be collagen (Table 4.4.1). As a result of heating, some of these proteins migrated out from the epimysial matrix and were designated as thermolabile protein. The release of thermolabile proteins was significantly influenced by the temperature ($P<0.0001$) and duration of heating ($P<0.0001$) (Table 4.5.1). Heating at 95 °C had favoured the release of more thermolabile proteins (~2% of raw weight) than heating at 80 °C (~0.9%) (Table 4.5.3). The longer the heating time the more proteins were liberated (Table 4.5.1) but the amounts released were <2.5% of the raw weight of epimysium (Table 4.5.3).

4.5.5 Pronase liberated protein

After heating, some of the amorphous collagen of the epimysium remained bound within the matrix and those proteins were extracted by employing pronase. Maturity of animals had no effect on the amorphous collagen released by pronase (Table 4.5.1) or in other words cow and heifer epimysium had produced similar amounts of amorphous collagen during heating at either 80 or 95 °C (Table 4.5.3). The length of heating and the temperature of heating had significantly influenced the amount of amorphous collagen produced (Table 4.5.1). Slightly higher amount of amorphous collagen was extracted after heating at 80 °C (~16%) than at 95 °C (~14%) (Table 4.5.3). As the length of heating time was increased, the pronase released amorphous protein content gradually decreased by about 1% of the raw weight of epimysium (Table 4.5.3).

Total amounts of protein liberated from epimysium after combined treatments of heating and subsequent hydrolysis with pronase was expressed as a percentage of total epimysial protein (Kjeldahl protein); *[(Thermolabile protein + Pronase liberated protein)/ Total protein*100.* This total protein released was influenced by the interaction, maturity*temperature (Table 4.5.1). According to the interaction means, cow epimysium had liberated about 37% of its proteins after heating at 80 and 95 °C but less amorphous proteins were extracted from heifer epimysium at 95 °C (42±7%) than at 80 °C (46±7%) (Table 4.5.2). In general, after heating at 80 and 95 °C (irrespective of heating time) and subsequent hydrolysis with pronase cow and heifer epimysium had retained about 63% and 54% of their proteins in the matrix. Perhaps, these proteins could be considered as 'thermostable protein' at 95 °C and after 1 h heating.

4.5.6 Shear stress

The resistance generated by a unit cross sectional area of epimysium (1 mm^2) to shear by a blunt blade was measured as shear stress. The raw epimysium from heifers and cows had shear stress values of 30.8±3 and 39.6±6.6 N/mm^2, respectively (Table 4.4.1). Epimysial shear stress was influenced by the maturity of animal (Table 4.5.1). For example, shear stress values of cow epimysium (6.5±2 N/mm^2) were twice as high as that of heifer epimysium (2.8±0.7 N/mm^2) after heating at 80 and 95 ºC (Table 4.5.3). The temperature of heating also had an effect on shear stress data (Table 4.5.1); at 95 ºC, epimysial shear stress was reduced more than at 80 ºC but the difference was small (Table 4.5.3). Further, long heating times also had significantly lowered the shear stress values of epimysium (Table 4.5.1) but a considerable reduction in shear stress was noted only between 15 min to 45 min heating (Table 4.5.3). The absence of two-way and three-way interactions (that included maturity) for shear stress data indicated that cow epimysium responded to heating time and temperature in the same manner as heifer epimysiusm did.

4.6 Discussion

Within the context of this study, epimysium was defined as the connective tissue sheath around the *longissimus* muscle, regardless of its thickness or macroscopic appearance. However, discrepancies were observed in the scientific literature on the definition of epimysium. Snowden et al. (1977) considered the connective tissue layer around the *longissimus* muscle as epimysium. King (1987) provided another definition; the connective tissue layer around the *longissimus* muscle with macroscopic bundles of parallel fibres was the tendon and the thin sheet of connective tissue that continues from tendon was the epimysium.

According to the scanning electron micrographs of the tissue used in the present study, fibres of heifer and cow epimysium were arranged as layers parallel to the long axis of epimysium.

Apparently, fibres of cow epimysium were more closely arranged than fibres of heifer epimysium. Intermittently distributed angular fibres were observed connecting those long fibres to develop a 'network' (Figure 4.3 a, of heifers and Figure 4.4 a and 4.4 b, of cows). Similar to the observations of this study, Kovanen (2002) had observed epimysial collagen as fibrous layers but perimysial collagen as wavy bundles. In contrast, another study had reported epimysial collagen as wavy bundles (Kragh et al., 2005). After 15 min heating at 70 ºC, an interior arrangement of woven fibre bundles was exposed in heifer epimysium. Palka (1999) made a similar observation after heating perimysium to temperatures above 60 ºC. Rowe (1974) also observed a similar arrangement of criss-cross collagen fibres in raw ovine perimysium. As such, the entrenched woven arrangement of collagen fibres might be a common phenomenon to both epimysium and perimysium. After 1 h heating at 55 ºC, collagen fibres from heifer epimysium were mostly denatured (Figure 4.3 c) and severe denaturation of fibres was noted after 15 min heating at 70 ºC (Figure 4.3 e). Cow epimysial collagen could withstand high temperature better than heifer epimysial collagen and a low degree of melting was noted after 1 h heating at 55 ºC (Figure 4.4 d) and 5 min heating at 70 ºC (Figure 4.4 e). Compatible with the observations of the present study, melting of collagen was reported after heating epimysium for 1 h at 60 °C and more extensive denaturation was reported after heating for an hour at 80 ºC (Wu et al., 1985).

According to the present study, *longissimus* epimysium from cows and heifers contained about 43.5 and 38.4% proteins, respectively, on a raw weight basis. Collagen content of *longissimus* epimysium from cows was about 39.5 and that of heifers was about 34.2% on a raw weight basis. Thus, collagen had contributed to about 91% of proteins in cow epimysium and 89% of the proteins in heifer epimysium. As such, epimysial properties might be mostly governed by the properties of collagen. Collagen content of epimysium was reported to vary with the muscle (type) and *pectoralis profundis* epimysium had about 22% collagen (on raw weight basis) (Light

and Champion, 1984). It was noted that analytical methods used in the present study were different from those used by Light and Champion (1984).

During aqueous heating, collagen dominant epimysium absorbed water and increased in weight. This phenomenon was opposite to the water release observed for myofibrillar proteins during heating (Bouton et al., 1976). Epimysial weight gain reflects the total amount of water absorbed by the matrix during heating while releasing some of its protein. Overall, cow or heifer differences (animal maturity) did not have any impact on epimysial weight gain during aqueous heating but this might be resulting from narrow margin of ages between cows (3-5 years) and heifers (<30 months).

At corresponding heating times at 55 ºC, epimysium had gained less weight than at 70 ºC. For instance, after 24 h heating at 55 ºC, weight of epimysium was increased by about 53% of its original weight and at 70 ºC, similar results (~54%) were observed only after 1 h heating. A significant increase in water uptake was noted as temperature was raised from 55 to 70 ºC. For example, epimysium had shown analogous values of weight gain (water uptake) after a short heating time at 70 ºC; after 540 min heating at 55 ºC (41±19%) and 45 min heating at 70 ºC (46±17%). Therefore, it was thought that these combinations of treatments had provided similar energy input.

Subjected to the more limited time treatments employed (a maximum of 60 min heating), temperature rise from 70 to 80 ºC offered no increase in water uptake and the results were similar to those observed at 70 ºC; cow epimysium increased its weight to about 37% and heifer epimysium had increased its weight to about 47.5% at 95 ºC. The only experiment comparable to the present study had measured the change in moisture content where after 35 min heating at 80 °C, moisture content of epimysium was increased by 170% (Field et al., 1970). Welke et al.

(1986) had reported 45% increase in epimysial weight after conventional oven cooking to reach the internal temperature to 71 °C. This observation matches the weight gain data of the present study despite the differences in cooking method.

Water uptake by proteins, in general, was influenced by the polarity of molecules and availability of sites for hydrogen bonding. Under the influence of heating, the observed increase in epimysial water uptake might be resulting from the dissolution of intermolecular bonds and unravelling of collagen structure to facilitate the entry and holding of water. It was clear from scanning electron micrographs that melting of collagen was already started after 15 min heating at 55 ºC and also after 5 min heating at 70 ºC. Therefore, it was evident that melting or denaturation of collagen had a positive effect on the water binding properties of epimysium. As observed in previous research, collagen lost its molecular stability with increasing hydration (Miles and Ghelashvili, 1999) due to breaking of hydrogen bonds and forming new water bridges within triple helices and reducing cohesive energy between polymer chains (Luescher, 1974; Batzer and Kreibich, 1981). Other research had confirmed that both thermal denaturation temperature and glass transition temperature of proteins (the temperature at which solidified amorphous protein become soft on heating or brittle on cooling) were reduced significantly with increasing hydration of protein, in particular as water content increased from 2 to 30%, the glass transition temperature of elastin was reduced from 195 to <10 ºC (Kakivaya and Hoeve, 1975; Bell and Hageman, 1996). Synthetic cross-linking of rat-tail tendon collagen also reduced the water uptake while increasing the thermal denaturation temperature (Miles et al., 2005). These workers had proposed that cross-linking brought collagen molecules closer and reduced the water retention ability between molecules. Contradictory to the latter hypothesis, it was observed in the present study that cow epimysium had higher amounts of pyridinoline cross-links before and after heating but water uptake data or to that matter even moisture content of raw epimysium were not different from that of heifers (having lower amounts of cross-links).

High epimysial water uptake observed at increasing temperatures and at increasing heating times had lead to the hypothesis that, the faster the energy supply during aqueous heating (in terms of high temperature at a constant heating time) the quicker the inter-molecular bond dissociation was. Also, the higher the energy supply was (longer heating times at a constant temperature) the higher the number of bonds dissociate.

The thickness change of epimysium (or connective tissue) during aqueous heating was reported here for the first time. As observed, the thickness increase of collagen dominant epimysium was not influenced by the maturity differences between cows and heifers at the temperatures 55, 80 and 95 ºC. However, 70 ºC was an exception where thickness of cow epimysium increased more than that of heifer epimysium and this deviation was significant, as all these experiments had used epimysium from the same random sample of animals. It was difficult to comment on the high thickness values of cow epimysium observed after 80 and 95 ºC temperature treatments due to the high standard deviations associated with them. The high standard deviations reported for thickness data showed the difficulty of using intact epimysium for research. Further, thickness (increase) data at any corresponding time treatment at 70 ºC were higher than data originating from 55 ºC temperature treatments but further increase in temperature had no clear effect on thickness increase. For example, after 15 min heating at 55 and 70 ºC, thickness change values of epimysium were 16±8, 110±64%, respectively. After 15 min heating at 80 or 95 ºC, the observed thickness change was 141±87%, as temperature had no effect on thickness change. Long heating times at 55 and 70 ºC had increased the epimysial thickness but the effect of heating time was not significant at 80 and 95 ºC. The latter observation had revealed that once the right input of energy was received biomolecules swell to the maximum and additional energy was dissipated.

Epimysial thickness change had represented a uniaxial deformation of epimysium and epimysium bound collagen because the other dimensions, length and width, had changed during heating. In early studies, bond scission and osmotic pressure were recognized as the two mechanisms of collagen swelling (volume expansion) in aqueous media (Elden, 1958). However, the observations made by Maroudas et al. (1973) on swelling of degenerating cartilage may have some relevance to the epimysial thickness increase observed in this study. According to Maroudas et al. (1973), hydration of cartilage was determined by the balance between swelling pressure of glycosaminoglycans and the restraining elastic forces of the collagen fibre network. When collagen fibre network was damaged (pathologically), the reduction in restraining elastic force was greater than the decrease in swelling pressure resulting from a loss of some glycosaminoglycans. Thus, the cartilage had absorbed water (Maroudas et al., 1973). Provided that the above explanation was true, the observed epimysial swelling or thickness increase might be resulting from thermal damage to collagen fibres facilitating water absorption. Glycosaminoglycans responsible for water uptake were tissue specific and their amounts vary with the age of the animal (Scott et al., 1994; Hayashi et al., 1995; Carrino, 1998; Wall et al., 1999; Carrino et al., 2003). Age related compositional differences in glycosaminoglycans of epimysium were not reported. As such, further investigation would be necessary to understand the causes of epimysial thickness increase.

Thermal shrinkage was considered as an inherent property of collagen. Both shrinkage and thickness increase were uniaxial changes to epimysium during heating and therefore did not explain swelling characteristic (volume change) of the collageneous tissue. According to the present study, the maturity of the animal had a significant effect on shrinkage; cow epimysium shrank more than the heifer epimysium. Also, SEM micrographs had shown that cow epimysium was more densly packed than the heifer epimysium. These observations closely

match with a previous observation (Wall et al., 1999). Accordingly, densely packed collagen shrinks more than loosely packed collagen.

As observed neither temperature of heating (80 and 95 ºC) nor heating time had any effect on the degree of epimysial shrinkage, measured after cooling. According to previous work, shrinkage of chordae tendineae (~98% collagen on dry basis) followed a sigmoidal pattern with heating time (measured in seconds while heating) under isothermal (from 65-90 ºC) conditions with and without an applied load. The heat-shrunken chordae tendineae slightly recovered within minutes during cooling to 37 ºC and then stabilized at the newly acquired length. This stabilization was thought to be resulting from the renaturation of some of the heat-denatured collagen (Chen et al., 1997; Chen et al., 1998). Therefore, the shrinkage data of the present experiment might represent the shrinkage after renaturation and stabilization of collagen. However, it was difficult to generalize observations made for chordae tendineae and apply to epimysium due to two reasons. Physiological functions of chordae tendineae (thread like fibrous tissue that connects tricuspid and mitral valves and also is attached to cardiac muscles) and epimysium (connects skeletal muscles and also participates in force transfer facilitating locomotion) were dissimilar. Secondly, epimysium had less collagen (38-44% protein on wet weight basis and ~90% of that was collagen) than chordae tendineae. The only previous study measuring epimysial shrinkage had reported a 16% reduction in length after 35 min heating at 80 °C (Field et al., 1970).

Numerous attempts were made over the years to understand the molecular kinetics during collagen shrinkage (Wöhlisch, 1932; Van Hook, 1947; Ramachandran and Kartha, 1955; Kirsch et al., 1998). According to one of the earliest hypotheses, collagen shrinkage represented fusion of crystalline structure (Wöhlisch, 1932 and Van Hook, 1947). X-ray patterns of shrunken collagen had shown that triple helices were not separated during shrinking but folded heavily

(Ramachandran and Kartha, 1955). According to Hayashi et al. (1995), thermal shrinkage of capsular collagen increases with increasing energy input during laser treatments. Electron micrographs of thermally denatured bovine tendon and shoulder capsular collagen had shown that the fibre diameter increased consistently with the linear shrinkage of tissue and finally, the tissue lost the fibrillar structure confirming denaturation (Kirsch et al., 1998; Wall et al., 1999). In another experiment, interfibrillar voids in ligament were absent after thermocoagulation of collagen at 67 °C (Ma et al., 2005). As thermal behaviour of isolated collagen did not represent that of matrix-attached collagen, studying a 'phenomenological model of shrinkage' was considered useful to understand the matrix effect (Wright and Humphrey, 2002). Studies on tissue models were not uncommon, i.e. Weir (1949) had studied the kinetic nature of thermal shrinkage of kangaroo tail tendon. The present study used bovine epmysium as a 'phenomenological model' (Wright and Humphrey, 2002) and demonstrated the effect of epimysial matrix on heat induced collagen shrinkage, water uptake, protein release and amorphous protein production.

An important phenomenon observed during aqueous heating of epimysium was protein release. Because proteins were liberated in response to heating, this fraction was designated as 'thermolabile protein'. At the two lower temperatures studied (55 and 70 ºC), the interaction between maturity*time were noted significant. The interaction means (at 55 and 70 ºC) indicated that with increasing heating times heifer epimysium had released more proteins than cow epimysium. As the temperature was raised to 80 and 95 ºC, heating time and temperature became determining factors of epimysial protein release where animal maturity had no effect. The overall impact of temperature on protein release was such that, the temperature rise from 55 to 70 ºC had increased the amount of thermolabile proteins released from epimysium but subjected to the limits of heating time (a maximum of 60 min), further increase in temperature (to 80 and 95 ºC) had no additional benefit. For example, the amounts of thermolabile proteins

released after 60 min heating were as follows; at 55 ºC about 0.64% for heifers and about 0.84% for cows, at 70 ºC about 2.3% for heifers and about 0.80% for cows, at 80 ºC about 0.87% and at 95 ºC about 2.1% for both cows and heifers.

The upper limit of protein release could be increased for heifer epimysium as energy input increases through long heating times. For example, after 24 h heating at 55 ºC (~14%) and 6 h heating at 70 ºC (~12%) similar amounts of epimysial proteins were released. This example also showed that, the higher the energy input was (high temperature) the more proteins were released within a short heating time and this was observed to occur for heifer epimysium. Another equally important observation was that, the upper limit of protein release for cow epimysium was not changed with the increasing input of energy, after 24 h heating at 55 ºC and also after 6 h heating at 70 ºC about 2% epimysial proteins were released.

The only comparable study conducted by Field et al. (1970) had reported protein release data similar to that of the present experiment. In which, *longissimus* epimysium from cows had released about 1.3% of its collagen after 1 h heating at 70 ºC (direct comparison of protein and collagen data are feasible because ~90% of epimysial proteins were observed to be collagen). However, the solubility of intra muscular connective tissue (perimysium and endomysium) was thoroughly investigated in the literature (Hill, 1966; Herring et al., 1967; Palka, 1999; Seidman, 1986). Some of these findings were highlighted below to set a foundation to understand solubility of epimysial collagen. After 1 h heating at 77 ºC, *sternomandibularis* muscles from heifers had released about 5% of their intra muscular collagen (Hill, 1966). From *longissimus* muscles of USDA grade A and B, about 10.5% and 9.4%, respectively, of intramuscular collagen became soluble after 70 min heating at 77 ºC (Herring et al., 1967). Another study carried out using *semitendinosus* muscles from young (18 months) bulls had shown that as internal temperature of meat had reached 70 ºC about 20% of intra muscular collagen were

released. However, protein release was diminished as temperature was increased beyond 70 ºC (80, 90, 100 and 120 ºC) (Palka, 1999). Solubility of intramuscular collagen from bull and steer loin muscles were found to be 17 and 16.5%, respectively, on a fat free weight basis (Seidman, 1986). According to Rochdi et al. (2000), cow epimysial collagen was more soluble (~65%) than cow intramuscular collagen (~20%) during heating at 90 ºC for up to 360 min.

As observed on SDS gels, during heating at 55 and 70 ºC epimysial collagen was mostly released as its component peptide chains, also known as single (α1 and α2 chains with a molecular weight of ~116 kDa) and double strands (2α chains with a molecular weight >200 kDa). These strands were thought to be originated from collagen type I since it was the dominant form in epimysium (Light and Champion, 1984). On visual examination, collagen bands from heifer epimysium were thicker than those from cow epimysium, an indication of release of more proteins and also an indication of thermolability of heifer epimysial collagen. Several other unidentified protein bands were observed after all treatments and specifically after 15 min heating at 55 ºC. Because the release of single and double strands of collagen was not evident (on SDS gels) at 15 min heating at 55 ºC, those unidentified bands must be of non-collagenous origin. There were no indications of hydrolysis of collagen bands to shorter chain peptides as temperature was raised to 70 ºC. Instead, accumulation of more single and double strands was evident as 116 and 200 kDa bands on SDS gels became wider and thicker. While highlighting that it was correct to compare relative thickness of bands from different treatments, it was also noted that these protein samples were exposed to 95 ºC (for 5 min) during sample preparation for SDS-PAGE (to increase solubility). That was a temperature higher than the original treatment.

Sufficient information was available in the literature to understand the products of collagen hydrolysis (Hayashi and Nagai, 1980; Light and Champion, 1984; Takahashi, 1988; Sini et al.,

1997). Collagen type I was known to produce α1, α2 and β11 and β12 bands on SDS gels but the separation of β bands (another name for 2α bands) were possible on low strength gels, i.e. 5-6% (Hayashi and Nagai, 1980; Sini et al., 1997). In addition to α1 and α2 from collagen type I, peptic digested epimysial collagen was also known to produce α1(III) from collagen type III (Light and Champion, 1984). Collagen from prawn epimysium also had produced α1, α2 and β chains after heating (Mizuta et al., 1999). Acetic acid hydrolysed collagen had produced α1, α2, β and γ chains (triple helices) (Takahashi, 1988). The collagen linked proteoglycan decorin was known to produce a band within the molecular weight regions 205-116 kDa (Sini et al., 1997). In general, it was clear that heating (55 and 70 ºC) could unravel collagen fibres in the epimysial matrix to liberate single and double strands but further hydrolysis was unlikely under the conditions of this experiment.

Based on the previous work (Snowden and Weidemann, 1978) it was assumed that heating would induce some of the epimysial collagen to undergo phase transition (to become amorphous) and it was also thought that some of the transformed collagen would remain attached to the matrix while thermolabile proteins were released. The amorphous collagen was measured as pronase digested product of heat-treated epimysium (free from thermolabile protein) because pronase was known to act only on amorphous areas leaving intact fibres unattended (Hörmann and Schlebusch, 1971; Snowden & Weidemann, 1978; Powell et al., 2000). It was also known that pronase act on C terminals of α chain to produce peptides of various lengths (Rosmus et al., 1967).

Pronase from *Streptomyces griseus* (a cocktail of enzymes) contained four proteolytically active fractions with different amino acid specificities (Trop and Birk, 1970). One of them was similar in function to pancreatic trypsin and another was similar to elastase. These enzymes were shown to be active on bonds between Gly-Val, Gly-Leu, Ala-Gly-Gly, Leu-Gly, Gly-Phr, Gly-

Ser, Lys-Val, Lys-Gly, Lys-Phe, Lys-Ser, Met-Gly and many other bonds (Trop and Birk, 1970). However, native collagen was insensitive to substrate non-specific enzyme hydrolysis. Consequently, pronase, pepsin, trypsin, chymotrypsin and elastase were reported to hydrolyse only the telopeptide areas of collagen (appendages to helical chain) and helical regions were unaffected. As a result, the optical rotation of enzyme digested collagen solutions were not changed after removing telopeptides through dialysis (Drake, 1966).

An important finding of this study was that the amount of epimysial amorphous collagen recovered by pronase (after heating at 55, 70, 80 and 95 ºC) was not related to the maturity of animals (cows and heifers). Longer heating times at 55ºC had a positive effect on the amount of amorphous collagen released by pronase but this relationship was not observed at 70 ºC. Hörmann and Schlebusch (1971) made similar observations; extended heating (30 and 60 min) at low temperatures (55 to 65 ºC) had increased the amount of amorphous collagen released by pronase. Evidently, increasing temperature beyond 55 ºC had a significant effect on the amount of amorphous collagen released by pronase and also on the speed of the process. For instance, about 14 and 16% of the epimysial proteins (on a weight basis) was extracted by pronase after 5 min exposure to 70 and 80 ºC, respectively. Similar amount of amorphous collagen was not extracted from epimysium even after 24 h heating at 55 ºC. Accordingly, increasing temperature from 70 to 80 ºC had increased the amount of amorphous collagen extracted by 2% and this might be resulting from the dissolution of high-energy bonds at the elevated temperature. As such, it was clear that the type of intermolecular bonds of collagen denatured during heating correspond to the input of energy. However, increasing the temperature of heating to 95 ºC had decreased the amount of pronase released amorphous protein by 2% (16% at 80 ºC and 14% at 95 ºC). As more thermolabile and less amorphous proteins were released at 95 ºC than at 80 ºC, it could be assumed that amorphous collagen was further changed to a thermolabile state as a result of higher energy supply at 95 ºC (when data expressed on the basis of protein g/100 g of epimysium). However, this hypothesis was not supported by the sum of

thermolabile and amorphous proteins presented on a protein basis; cow epimysium had produced the same amount of total proteins (thermolabile+amorphous proteins) at 80 and 95 ºC but heifer epimysium had produced more total proteins (thermolabile+amorphous proteins) at 80 ºC than at 95 ºC.

Collagen exposed to thermal treatments undergoes phase transition, which involves melting of crystalline (like) regions (Flory and Garret, 1958). This phase transition began in the heat labile domains near the C-terminus of collagen molecules that were located in the gap regions of the quarter-staggered arrangement (Miles et al., 1995). According to a theoretical model of collagen melting, collagen molecule had domains of variable molecular stability and such domains were located closer to the carboxy terminus, which was deficient in hydroxyproline (Miles and Bailey, 2001). As Okamoto and Saeki (1964) reported, collagen from dehydrated bovine Achilles tendon had three levels of structural organization; (a) an unstable crystalline region with little orientation which melted between 80–180 ºC, (b) a natural amorphous region which underwent second order phase transition at 120 ºC and (c) a stable crystalline region with a clear melting point at 200 ºC, which was thought to be stabilized through ionic-bonds and high degree of cross-linking. Keeping in line with the observations listed above, the differences observed in epimysial protein release at different energy inputs (provided as time and temperature) suggested the progressive dissolution of high-energy bonds. This was compatible with a previous hypothesis that collagen had no unique thermal denaturation temperature but transition takes place over a wide range of temperatures (Wright and Humphrey, 2002)

The low values observed for amorphous collagen content (a maximum of 16% on a weight basis) after heating at 70, 80 and 95 ºC, could be attributed to two causes. As explained by Lepetit (2008), helix to coil transition happened (to form amorphous collagen) when isolated collagen fibres shrink >75% of their original length. Therefore, one likely reason for the low

amounts of amorphous collagen produced was the inability of epimysial collagen to shrink freely due to a matrix effect; for example, cow and heifer epimysium shrank only about 68% and 46%, respectively. The other likely reason for the low yield of amorphous collagen was the renaturation of coils during cooling (prior to pronase hydrolysis) as explained by Hörmann and Schlebusch (1971). Accordingly, the longer the duration of exposure to 4 ºC, the more the transformation of coils to helices was. As a result, pronase could hydrolyse only the irreversibly denatured collagen. If interpretation of Hörmann and Schlebusch (1971) is correct, the amorphous collagen extracted during the present experiment represented only the irreversibly denatured epimysial collagen. These authors also noted that the maximum amount of amorphous collagen could be recovered after 60 h incubation with pronase at 4 ºC. In the present experiment, enzyme digestion was carried out at room temperature and for about 16 h and therefore, it was unlikely that the incubation time had any significant effect on the low amounts of amorphous collagen produced due to the high temperature used.

EC content in the pronase-released collagen was measured as an indicator of the pyrrolic cross-links in order to understand their presence in amorphous regions of collagen molecules. As noted in the present study, none of the variables tested (animal maturity, heating time and temperature- 50 and 70 ºC) had influenced the amount of EC/g of pronase-liberated protein. Unlike EC cross-links, pyridinoline and deoxypyridinoline cross-link content varied with the animal maturity where epimysium from cows had more cross-linking than epimysium from heifers. This observation was compatible with previous observations made that pyridinoline content was observed to increase with the maturity of animals. However, human tissues were an exception, cross-link content began to decline for people over 30 years of age (Moriguchi and Fujimoto, 1978; Smith and Judge, 1991). As observed, some of the pyridinoline and deoxypyridinoline cross-links might be located in thermolabile areas of collagen molecules

because heifer and cow epimysium had liberated about 74.2 and 66.4 % of their cross-links, respectively, during aqueous heating at 70 ºC.

It was necessary to focus on the similarities and differences between pyridinoline and EC cross-links as both were released after heating. EC cross-links were detected in collagen Type I, III and IV and also in elastin (Scott et al., 1981; Scott et al., 1983; Kemp and Scott, 1988; Kuypers et al., 1992). EC was a trivalent pyrrole, formed through a reaction between a lysine aldehyde in telopeptide region (both C and N terminals) and a bivalent keto-amine cross-link (Scott et al., 1981; Kuypers et al., 1992). EC and pyridinoline cross-links were formed through the same pathway, except that hydroxylysine participated in pyridinoline cross-link formation (Kuypers et al., 1992). Pyridinolines were confined to both C and N terminals of telopeptides but 85% EC are limited to N terminals of telopeptides (Hanson and Eyre, 1996). Further, a cross-linking site was located on the themolabile domain of collagen at the C terminus (Miles and Bailey, 2001). Perhaps, both EC and pyridinoline cross-links were located in the thermolabile areas of collagen molecule. Based on these findings several conclusions were made: (a) detection of EC cross-links in amorphous collagen and release of pyridinoline during heating indicated that they were located in the heat sensitive regions of collagen fibres; (b) epimysium from animals in the age group of about 30 months (heifers) to 5 years (cows) had similar amounts of EC cross-links/g pronase protein but (c) pyridinoline cross-link present in g of protein increased with animal maturity.

A considerable difference was observed between shear stress values of raw epimysium from cows (~39.6 N/mm^2) and heifers (~30.8 N/mm^2). As noted in the present study, shear stress values decreased with the maturity of animals after temperature treatments at 55, 70, 80 and 95 ºC. The effect of animal maturity on shear stress was prominent at 70 ºC. For example, shear stress values of heifer epimysium (2.1±0.7 N/mm^2) were 5 times lower than those of cow

epimysium (11.6±6 N/mm^2) after heating at 70 ºC. At 55 ºC and also at 80 or 95 ºC the epimysial shear stress gap between cows and heifers was a two-fold difference. The reason was obvious that the thermal transition or denaturation of proteins had just begun at 55ºC/15 min treatment and shear stress values demonstrated the relative differences observed in raw epimysium. As temperature was increased to 70 ºC, it might be that thermolabile bonds in heifer epimysial proteins were (almost) completely disintegrated but not those in cow epimysium. This might have lead to the largest shear stress gap observed at 70 ºC. As temperature was increased (to 80-90 ºC), shear stress values of cow epimysium were decreased (perhaps, some bonds requiring high energy input to disintegrate were attacked). However, shear stress of heifer epimysium had reached the lowest values with aqueous heating at 70 ºC and remained unchanged during further heating at 80 and 90 ºC.

In addition, long heating times (at a constant temperature) and temperature increase (at a constant heating time) also had contributed to the shear stress reduction. For instance, the observed shear stress values after 60 min heating at 55, 70 and 80 or 95 ºC were ~21±8, 7.3±4.7, and 3.6±2.8 N/mm^2. Another interesting observation was that shear stress values of epimysium had reached to a similar value (~5 N/mm^2) after 3 h heating at 70 ºC and 5 min heating at 80 and 95 ºC. It was logical to assume that these combinations of time and temperature had provided similar energy input that lead to bond scission and subsequent reduction in shear stress. Therefore, it is proposed that the same degree of reduction in shear stress can be attained through using different combinations of time and temperature. For example, long heating times at low temperatures and short heating times at high temperatures should be able to reduce epimysial shear stress to the same degree. However, the above reasoning was not supported by the pronase liberated protein data. As noted elsewhere in this discussion, pronase liberated protein (amorphous collagen) content was not affected by the maturity of the but shear stress was always influenced by maturity of animals. In other words, at temperatures above 70 ºC,

similar amounts of amorphous collagen were extracted from cow and heifer epimysium but shear stress values of cow epimysium were always higher than those of heifers. It was also observed that shear stress values of cow epimysium decreased as temperature increased from 55 to 95 ºC while shear stress values of heifer epimysium reached a status quo after 70 ºC. The observed negative correlations between shear stress and pronase released proteins for cows (r=-0.85) and heifers (r=-0.54) did not explain the observed phenomenon. As observed by Hörmann and Schlebusch (1971) and as already discussed, it was possible that amorphous collagen measured in the current experiment represented only the irreversibly denatured collagen excluding renatured collagen. Also, it was possible that renatured collagen have had weak inter molecular bonds after renaturation due to incorporation of water molecules into proteins. Inclusion of water molecules might have contributed to the observed shear stress decrease in both cow and heifer epimysium with the supply of heat energy. Therefore, the original hypothesis set, 'epimysial shear stress reduction during heating is a result of collagen phase transition', cannot be verified by measuring only the pronase soluble collagen unless the renaturation process is stopped. A different measurement, insensitive to renaturation or in other words sensitive to weakened collagen molecules and/or increased number of water molecules would be necessary to test the hypothesis. The observed reduction in shear stress of cow and heifer epimysium could be attributed partly to the disintegration of hydrogen bonds of collagen and partly to the disintegration of cross-links as observed for EC and pyridinoline. Higher shear stress values of cow epimysium (than heifer epimysium) after heating at 95 ºC, was a clear indication for the presence of some heat stable bonds in cow collagen, perhaps pyridinolines.

Strong, negative correlations were observed between shear stress values and thickness change (cow, r=-0.89; heifer r=-0.71) and also between shear force and weight gain (cow r=-0.78; heifer r=-0.74). However, these parameters could not explain the reason for the observed difference in shear force between cow and heifer epimysiusm. Further, epimysial weight gain was not

affected by the animal maturity at 55 and 70 ºC (according to the statistical model used) but temperature increase had favoured weight gain. Temperature increase to 80 and 95 ºC did not increase epimysial weight gain over and above values reported at 70 ºC. Epimysial thickness change was not affected by the animal maturity at 55, 80 and 95 ºC. At 70 ºC, thickness of cow epimysium was increased more than that of heifer epimysium and at 80 and 95 ºC a similar trend was noted (P=0.075); high standard deviations of thickness data might have influenced the lack of statistical significance. Even though, weight gain and thickness change were not related to animal maturity at all temperatures studied, shear stress was related to animal maturity; cow epimysium had higher shear stress values than heifer epimysium.

It was imperative to consider the combined effect of thermolabile proteins plus amorphous proteins (on a protein basis) on shear force. Accordingly, after 1 h heating of cow and heifer epimysium at 70 ºC, about 40±10% denatured proteins were extracted. After 1 h heating at 80 ºC, about 46±7% and 37±4% denatured proteins were removed from cow and heifer epimysium, respectively. Therefore, heifer and cow epimysium had retained about 54 and 63% of their proteins in the residue after heating at 80 ºC. (It was important to note that heating time had no effect on total protein release at 80 and 95 ºC). Subject to the high standard deviations (noted), the amounts of proteins retained in cow and heifer epimysium may be considered similar. Under the same conditions of heating, shear stress of heifer epimysium (2.78± N/mm^2) was twice as low as that of cow epimysium (6.5±2.2 N/mm^2). As such, it was concluded that the amount of thermolabile plus amorphous proteins or their reciprocal heat stable proteins retained in epimysium (subjected to the limits of the experiment) were not responsible for the shear force differences observed between cows and heifers. Even though, some pyridinoline cross-links were released during heating, cow epimysial residue had retained more cross-links than heifer epimysium after their exposure to 70 ºC for 3 h. Perhaps, unreleased pyridinoline or other (unknown) kinds of cross-links may have contributed to the high shear stress values. Similar

hypotheses were earlier proposed; accordingly, thermal stability and tension of denatured collagen was arising from mature multivalent cross-links and mostly from pyridinoline cross-links (Bailey et al., 1977; Lous et al., 1982; Bailey, 1989; Smith and Judge, 1991). The draw back of the experiment leading to the latter hypothesis was that these authors assumed that cross-links were explicitly heat resistant and therefore, total cross-link content was measured and considered as an indicator of thermal stability. Other researchers found no relationship between the type of cross-link and toughness (Shimokomaki et al., 1972; Horgan et al., 1991; Avery et al., 1996). Proving that pyridinoline cross-links (or any other cross-link to that matter) were the true cause of higher shear stress values of cow epimysium could be attained through studies on gene deficient (for the selected trait) animals and which was beyond the scope of the discussion of this study.

It was necessary to discuss the causes of high standard deviations observed for parameters measured. According to the data for individual animals, it was clear that out of four cows studied, two cows had similar epimysial properties. Epimysial properties of one cow were very close to that of heifers. Epimysial properties of the fourth cow were higher than that for other three cows. This led to the wide distributions in data. It was also noted that the meat (that was used to extract epimysium) originated from animals from various herds of unknown backgrounds. Thus, the differences in animal management practices, feed quality, number of pregnancies and so on also might have contributed to the high standard deviations.

On the whole, it was observed that cow epimysium always had higher shear force values than heifer epimysium. But, shear force of heifer epimysium could be substantially decreased (~2.1 N/mm^2) by a short exposure to temperatures above 70 ºC. If renaturation was the major cause for the low amounts of amorphous collagen produced then pronase hydrolyis of amorphous collagen might be inadequate to build the relationship between the true amounts of amorphous

collagen produced (during heating) and shear stress. Then, it was critical to prevent renaturation. However, if epimysial matrix effect was the major cause for the low amounts of amorphous collagen produced (collagen was not free to respond to heat as bound to the matrix), then this experiment represented the true relationship between amorphous collagen produced after epimysial heating and associated shear stress decrease.

4.7 Summary and conclusions

Protein was a major constituent of *longissimus* epimysium from cows (44%) and heifers (38%), with collagen comprising about 90% of epimysial proteins. Physicochemical properties of epimysium should, therefore, be governed by the properties of collagen. The 'maturity of animal' generally had no clear effect on the epimysial properties assessed, except for shear stress. At all times, shear stress values of cow epimysium were higher than that of heifer epimysium. Animal maturity had an effect on epimysial thickness only at 70 ºC. Heifer epimysium had released significantly high amounts of thermolabile proteins than cow epimysium after long heating treatments at 55 and 70 ºC. The amount of amorphous collagen extracted by pronase was independent from animal maturity. Pyridinoline cross-link content of epimysium was increased with the maturity of animals. However, EC cross-link/g of pronase protein did not differ between two maturity groups. A brief exposure (5 min) to 70 ºC was sufficient to significantly modify epimysial proteins to increase weight gain, thickness, thermolabile proteins and amorphous collagen contents. Similar changes in measured parameters could be obtained at 55 ºC after long durations of heating. The temperature increase beyond 70 ºC (subject to the temperature upper limit of 95 ºC) either had a marginal improvement or no further improvement at all on measured parameters except for shear stress.

Thermolabile proteins consisted of both single (α) and double (2α) strands of collagen. The observed upper limit for the extractable amorphous collagen was 16% on a weight basis. After prolonged heating at 80 and 95 ºC, the release of the amorphous collagen was slightly decreased. The residue of cow epimysium contained more pyridinoline cross-links than that of heifer epimysium after 3 h heating at 70 ºC. Because some of the EC cross-links were released with amorphous collagen and some pyridinoline cross-links were released with thermolabile proteins, they must be located in thermolabile areas of collagen molecules. Temperature increase beyond 70 ºC had no effect on the shear stress decrease of heifer epimysisum. Shear stress values of cow epimysium were decreased with the increasing temperature from 55 to 95 ºC and also with long heating times. The amount of amorphous collagen produced during heating of epimysium did not determine the degree of connective tissue driven toughness measured as shear stress.

Based on the above summary several conclusions were made; (a) cow epimysial proteins contained bonds requiring high energy to unravel, (b) epimysial shear stress differred with the maturity of animals, (c) within a maturity level, different combinations of temperature and heating time which provided the same energy level, brought the same degree of change such as reduction in shear stress, increase in water uptake, thickness, thermolabile and amorphous protein, (d) the amount of amorphous collagen extracted by pronase (after cooling to 4 °C) was independent from animal maturity (e) changes in amorphous collagen content during heating did not explain changes in shear stress.

4.8 Connection to the next study

As observed in the preceding work, shear stress of heifer epimysium was brought down to a minimum value (2.1±0.6 N/mm^2) at 70 °C and further reduction was not attained through

increased temperature. It was also noted that, cow epimysial shear stress values were always higher than that of heifer epimysium but could be reduced further by heating to higher temperatures. These observations highlighted the likelihood of cow epimysial proteins to contain high-energy intermolecular bonds while admitting the absence of similar bonds in heifer epimysium. To meet the overall objective of this project, it was important to have strategies identified to further decrease shear stress of cow epimysium to the minimum values reached by heifer epimysium. Therefore, the next study was planned to investigate the usefulness of strong and weak acids and alkali to disrupt intermolecular bonds in cow epimysium.

5. THERMAL MODIFICATION OF STRUCTURAL INTEGRITY OF COW EPIMYSIUM TREATED WITH STRONG AND WEAK ACID AND ALKALI

5.1 Abstract

This study investigated the effects of strong (HCl) and weak (CH_3COOH) acids and strong (NaOH) and weak (NH_4OH) alkali on the physicochemical properties of cow epimysium to assess the efficacy of different treatments in decreasing the shear stress of epimysium during heating. As indicators of modified epimysial structure, percentage weight gain, percentage thickness change, protein (g/100g) and free amino groups released (mg/g) were measured in addition to shear stress (N/mm^2). Compared to the values of raw epimysium, shear stress was considerably reduced after long pre-equilibration times in HCl and CH_3COOH and subsequent heating to 70 ºC, after exposing to increasing concentration of HCl and also after heating at 70 ºC with increasing concentration of NaOH. The concentration of NH_4OH or pre-equilibration treatment in it could not decrease epimysial shear stress. Instead, NH_4OH had an epimysium stabilization effect at 55 ºC. Changes in other physicochemical properties measured (protein release, thickness and weight gain) showed that some structural changes took place in epimysium after treatments but were not substantial indicators to describe the degree of reduction in epimysial shear stress.

5.2 Introduction

The contribution of connective tissue to beef toughness was extensively investigated (Sifre et al., 2005; Purslow, 2005; Lepetit, 2007). Perimysial and endomysial collagen content and collagen fibre diameter were reported to correlate with beef toughness (Light et al., 1985). Shear force originating from intramuscular connective tissue also was a good indicator of tenderness of beef (Møller, 1981). To reduce the connective tissue driven toughness, beef was marinated with organic acids such as acetic, lactic and citric (Arganosa and Marriott, 1989; Aktas and Kaya, 2001; Berge et al., 2001). The application of acids to tenderize meat might be linked to their ability to solubilize collagen (Bowes et al., 1955; Kruggel, et al., 1970; Whiting and Strange, 1990; Meyer, 2003). Acetic acid (CH_3COOH) *per se* was often used for the isolation of collagen from tissues (Maekawa et al, 1970; Rhodes and Miller, 1978; Nagai and Suzuki, 2000). The relationship between alkali and tenderness of meat was scarcely studied. According to Hamling and Calkins (2008 a), a mixture of ammonium hydroxide and salt could reduce the shear force of beef. In other research, insolubility of collagen in alkali was considered as a measure of stability of connective tissue (Fremery and Streeter, 1969; Bayne et al., 1971).

Both acids and alkali were known to influence the thermal denaturation temperature of collagen (Miles et al., 1995; Friess and Lee, 1996; Hattori et al., 199; Góes et al., 2002; Mu et al., 2007). Heating collagen in a solution of 0.5 M acetic acid had decreased the thermal denaturation temperature of collagen to 40 ºC, compared to 62 ºC observed after heating in water (Miles et al., 1995). This reduction was attributed to the low levels of activation energy required for the thermal transition. A peptide residue of 66 amino acids, located in the gap zone of quarter-staggered collagen molecules, was reported to unwind after the acetic acid treatment and a residue of 26 amino acids was noted unwinding after heating in water (Miles et al., 1995). The effect of acid in lowering thermal denaturation temperature was verified through both

differential scanning calorimetry (DSC) and fourier transform infrared spectroscopy (FTIR) studies; acetic acid soluble collagen (pH 3.5) was denatured at 43 ºC (Friess and Lee, 1996). After acetic acid treatment, soluble collagen had produced 2 peaks; the peak at ~32 ºC was attributed to "defibrillation" and the other at 40 ºC peak was attributed to denaturation of collagen (Mu et al., 2007). The effect of alkali on collagen was similar to that of acids; for example, the thermal denaturation temperature of collagen treated with 3% sodium hydroxide (for 20 days) was decreased to 35 ºC but without altering the triple helical conformation (Hattori et al., 1999). The decrease in the thermal denaturation temperature was proportional to the length of the alkali treatment (Góes et al., 2002). Sodium hydroxide treatment was also reported to induce deamination of asparagines and glutamine to produce aspartic acid and glutamic acid (Hattori et al., 1999; Radhika and Sehgal, 1996). Anionic collagen, produced by alkali treatments, had high piezoelectric properties (ability to acquire a charge during squeezing and twisting) and it was attributed to increased molecular organization (Góes et al., 2002). Acid and alkali extracted collagen from bones had similar properties except that, alkali extracted collagen was rich in α1 and α2 chains but acid extracted collagen was rich in 2α chains. It was proposed that alkali breaks intermolecular bonds to liberate α1 and α2 chains (Kemp and Tristram, 1971).

The effect of strong acids and alkali on isolated collagen was previously studied (Knaggs, 1929; Theis and Jacoby, 1943; Bowes and Kenten, 1950) but the response of epimysium and other intact connective tissue to acid/alkali treatments was not investigated. Because the isolation procedure had modified the protein and the matrix effect was eliminated, such experiments may not represent the response of intact tissue. This research was planned to explore the influence of acid and alkali on epimysial physicochemical properties with the objective to decrease the shear stress of cow epimysium to <2 N/mm^2, because it was noted in a previous experiment that shear force of heifer epimysium was decreased to about 2 N/mm^2 during aqueous heating at 55 and 70 ºC (section 4.4.6). No complete study was reported before assessing the combined effect of

temperature, concentration of strong and weak acids/alkali and pre-equilibration of epimysium (or isolated collagen for that matter) in acid/alkali.

The hypotheses leading to the present study were as follows; (a) a strong proton donor has an increased capacity to weaken the epimysium structure than a weak proton donor through the hydrolysis of bonds of the collagen and (b) completely ionized alkali has an increased capacity to weaken the epimysium structure than an alkali that dissociates weakly.

5.3 Materials and Methods

5.3.1 Sample preparation and acid/alkali heating

Bovine *longissimus* muscles from eighteen cows of utility grade, the lowest rating for quality, were purchased from a local supplier between 7th and 10th day of post-mortem and stored at 4 °C. On the 10th day post-mortem, the dorsal epimysium was excised with about ½ cm thick layer of muscle. Epimysium samples were stored at -20 °C after vacuum packing, until further used. Prior to use, epimysium was thawed overnight at 4 °C and visible fat and muscle fibres were removed using a scalpel blade. Two strips, each 2 cm wide, were cut along the length of the epimysium from the vertebral column side and then the strips were further divided into 2x2.5 cm pieces for the random application of treatments. The weight and the thickness at three different locations on a piece of epimysium (Dial Thickness Gauge No: 7301, Mitutoyo Corporation, Japan) were immediately recorded and samples were placed in 50 mL polycarbonate tubes. Two pieces (2x2.5 cm) of epimysium from each cow were stored at -20 °C for later determination of raw shear stress.

Epimysium pieces obtained from a cow (considered as block in statistical analysis) were randomly divided into two groups and assigned to either of 55 or 70 °C temperature treatments.

Each epimysium piece within a temperature treatment was immersed in 10 mL of one of the concentrations of an acid or base. HCl, CH_3COOH and NH_4OH were used at 0.1, 0.25 and 0.5 M concentrations. NaOH was used at 0.01, 0.025 and 0.05 M concentrations. The pH values of HCl and CH_3COOH solutions were in the ranges of 1.10-1.54 and 2.99-3.21, respectively. The pH values of NaOH and NH_4OH solutions were in the ranges of 12.0-12.70 and 11.12-11.40, respectively. Epimysium pieces also received one of the three pre-equilibration treatments in acids or alkalis for 0, 90 or 180 min. At the end of equilibration with acid/alkali, samples were brought to the desired temperature in a pre-heated water bath and maintained 15 min at that temperature. Immediately after heating the aqueous phase was decanted into 30 mL polycarbonate centrifuge tubes and allowed to cool at room temperature. After neutralizing the pH with HCl or NaOH as appropriate, the tubes were centrifuged at 31,000x*g* (J2-HC centrifuge, JA-17 rotor, Beckman Instruments, USA) for 30 min at 20 °C. Then the volume of the aqueous phase was adjusted to 10 mL with de-ionized water and stored at -20 °C for the determination of protein and OPA sensitive amino groups. The epimysium pieces collected after the heat treatment were stored at 4 °C to determine shear stress and weight the following day. The effects of two acids were tested with six replicates and the effects of two alkalis were tested with three replicates.

For the comparison of the efficacy of acid and alkali treatments against water treatment, a parallel experiment was carried out using epimysium samples obtained from the same cows. Epimysium pieces of 2x2.5 cm, prepared as already explained were heated to either 55 or 70 ° C for 15 min with 10 mL of water. Each treatment was repeated with epimysium obtained from the same cows as used for each of the acid/alkali experiments.

5.3.2 Proximate analysis

Epimysium from each animal remaining after sampling was frozen to -20 °C and grinding carried out according to the procedure previously described in section 4.2.2. To characterize basic chemical properties of epimysium, total moisture (AOAC 950.46 B, 1990), crude protein (AOAC 981.10, 1990) and crude fat (AOAC 960.39, 1990) were determined in duplicate.

5.3.3 Determination of shear stress

Shear stress of acid and alkali treated epimysium pieces were determined according to the procedure described in section 4.3.10. The maximum force generated on a unit cross sectional area of epimysium and corrected for raw thickness was presented as shear stress (N/ mm^2).

5.3.4 Determination of protein

Proteins liberated after acid, alkali and water treatments were determined according to the BCA protein assay (Smith et al., 1985) as previously described (section 4.3.6).

5.3.5 Determination of O-phthaldialdehyde (OPA) reactive free amino groups

OPA reactive free amino groups, liberated from the epimysium as a result of acid, alkali and water treatments (except with NH_4OH) were determined according to Church et al. (1985) and Wanasundara et al. (2002). The OPA reagent was a mix of 5.7 mM DL- dithiothreitol, 0.1 M sodium tetraborate decahydrate, 1% (w/v) sodium dodecyl sulfate and 6 mM phthaldialdehyde. The latter was first dissolved in a minimum possible volume of 97% (v/v) ethanol. A fresh reagent was prepared each day before analysis. An aliquot of 400 µL of an appropriately diluted sample was mixed with 3 mL of OPA reagent. Then the mixture was allowed to react for 2 min at room temperature and absorbance spectra was measured at 340 nm (UV-VIS spectrophotometer, Genesys 5, Milton & Roy Company, USA). Glycine was used as the

standard. This was a modification introduced to the original method. The amount of OPA reactive free amino groups was calculated as glycine moieties. Because, NH_4OH interfered with the detection method, amino groups were not measured in NH_4OH treated samples.

5.3.6 Electrophoresis

The pH neutralized aqueous phase (obtained after heating epimysium at both 55 and 70 ºC with acid/alkali with a 180 min pre-equilibration treatment) preserved at –20 °C was thawed to room temperature. To obtain 50 µg of dry protein from selected treatments required volumes were computed based on the BCA protein assay data. Then samples were lyophilized to obtain a dry protein. The sample preparation procedure and electrophoresis were as described in the section 4.3.7.

5.3.7 Supplement

The following experiments were carried out on epimysium from selected animals and also on selected acid and alkali treatments in order to facilitate understanding on some of the changes observed after original treatments.

5.3.7.1 Differential scanning calorimetry

Raw epimysium from 6 cows was first cut into small pieces and then finely ground after freezing in liquid N_2. About 0.5 g of ground epimysium was weighed into screw capped glass tubes. Then, sets of 6 tubes were treated with 10 mL of one of the following liquids; de-ionized water (control), 0.25 M HCl, 0.25 M CH_3COOH, 0.25 M NH_4OH or 0.025 M NaOH and equilibrated overnight at 4 ºC. Excess liquid was drained out and epimysium samples were briefly dried on filter paper. About 7-8 mg of samples were weighed into coated aluminium pans and hermetically sealed with lids (catalogue numbers 900796.901 for pans and 900790.901 for lids, TA Instruments, Delaware, USA). A differential scanning calorimeter (DSC Q 2000,

TA Instruments, Delaware, USA) equipped with an inbuilt cooling system and an auto sampler was used for the thermal analysis. Sealed, empty pans were used as the reference. Heating rate was 5 ºC/min over the range of 20-140 ºC. Peak thermal denaturation temperature was determined using curve integration software (TA Universal Analysis 2000).

5.3.7.2 Fourier transform infrared spectroscopy (FTIR)

FTIR was performed on cow epimysial tissue pre-conditioned with selected acid and alkali treatments. A cross-sectional band of about 7.5 cm wide was cut from the anterior end of epimysium and separately stored at -20 ºC. On the day of processing epimysium was first thawed to 4 ºC and then visible fat and muscle fibres were scrapped off using a scalpel. Epimysium was diced into 2x2.5 cm pieces and each piece was placed in a polycarbonate tube. Heating was carried out in duplicates. Two samples were added with 10 mL of de-ionized water and then immediately brought to an internal temperature of 70 ºC in a pre-heated water bath. Heating was continued for 15 min. Epimysium heated in water was considered as the control. Either 10 mL of 0.25 M HCl or 10 mL of 0.5 M NH_4OH was added to another 2 samples and these were pre-equilibrated for 180 min, at room temperature, prior to heating as mentioned above. Ten millilitres of de-ionized water was added to another two samples and these were heated at 120 ºC for 20 min at 20 psi. At the end of the heating liquid from each tube was drained and the epimysium samples were stored at -20 ºC. Selected samples were trimmed and sectioned to fit the mould for cryopreservation. The plastic moulds carrying the specimens were filled with embedding compound OCT cryomatrix® (Shandon Company, Pittsburgh, USA) and the entire preparation was frozen in liquid nitrogen. Serial sections of 6 μm in thickness were cut from cryo-preserved tissue at -20^{o}C using a cryo-microtome. Sections were placed on reflective-coated microscope slides (Low-e Microscope Slides, Kevley Technologies, Chesterland, OH) for spectroscopic analysis.

The FTIR imaging study was conducted at the Canadian Light Source using the 01B1-1 Mid IR beam line fitted with an IFS66v/s interferometer and Hyperion 2000 Microscope using a single point MCT detector. Spectroscopic mapping was performed in reflection mode with diffraction-limited spatial resolution. The confocal aperture size was set at 10μ and the sample was raster scanned under a 36x Schwarzschild objective. For each pixel, 64 scans, with a 4 cm^{-1} spectral resolution, were co-added. Interferograms were converted to spectra by applying a Fast Fourier Transform with a Blackman-Harris 3-Term apodization function, and also with a zero filling factor of 2. The frequency of infrared spectra examined was between 800 and 2000 cm^{-1}. The data was analyzed using computer software OPUS (Bruker Optics, Reinstetten, Germany).

5.3.7.3 Transmission Electron Microscopy (TEM)

Another set of epimysium samples was prepared with 0.25 M HCl, 0.5 M NH_4OH and water following the same procedure described previously (section 5.3.7.2). Immediately after heating epimysium was diced into ~2x2 mm pieces and preserved in 3% gluteraldehyde and 0.1 M sodium cacodylate buffer at 4 ºC. Samples were shipped to the Advanced Microscopy Facility of Department of Biological Sciences, University of Alberta, Canada for microscopic imaging. Dehydration of samples was carried out in a gradient of mixtures of ethanol/water and also in absolute ethanol. Samples were embedded into Spurr's resin. Using a Reichert-Jung Ultramicrotome, 50-60 nm thick sections were cut and placed on 300 mesh grids. The sections were then stained with uranyl acetate and lead citrate. The grids were examined with a Philips Morgagni 268 Transmission Electron Microscope.

5.3.8 Statistical analysis

The effects of HCl, CH_3COOH, NaOH and NH_4OH on epimysium properties were independently evaluated. The other variables (temperature, concentration and pre-equilibration time) were allocated in a factorial arrangement and data were analyzed in a split-plot design

using PROC-GLM of SAS 9.1 programme (SAS Institute Inc, USA). The pre-determined significance level for this study was $P<0.05$. An epimysium piece was the experimental unit and animal served as the blocking factor. The main-plot treatment temperature had two levels, 55 and 70 °C and each sub-plot treatment had three levels: pre-equilibration (no equilibration, 90 and 180 min pre-equilibration) and acid/base concentrations (0.1, 0.25 and 0.5 M or 0.01, 0.025 and 0.05 M as appropriate). Main effects of treatments and their two-way and three-way interactions were included in the model. Mean separations were performed using the least significant differences procedure of SAS programme. A two sample paired T test was carried out to compare the efficacy of acid/alkali treatments (at 0 min pre-equilibration treatment) against that of water using PROC-T TEST of SAS 9.1 programme.

5.4. Results

The pH values of the acids and alkalis used were reported to understand the pH effect on physicochemical properties of epimysium. As concentrations of HCl and CH_3COOH were increased from 0.1 to 0.5 M, pH values were changed from 1.54 to 1.10 (for HCl) and 3.21 to 2.99 (for CH_3COOH). Three different concentrations of NaOH, 0.01, 0.025 and 0.05 M had pH values ranging from 12 to 12.70. Three different concentrations of NH_4OH, 0.1, 0.25 and 0.5 M used resulted in pH values ranging from 11.4 to 11.12. The possible buffering effect of epimysium after acid and alkali treatments was ruled out as pH values of acid and alkali extractions separated after epimysial heating were not significantly changed. (The pH values of acid and alkali extractions were measured for every sample before neutralization to prepare them for chemical analysis. Data are not shown).

5.4.1 Results of acid treatments

Basic physicochemical characteristics of cow epimysium were listed in Table 5.1. Accordingly, average protein content of epimysium was 46±4% and fat content was 4±2%. Epimysium

Table 5.1 Characterization of cow epimysium on moisture, protein, fat and shear stress

	Mean ±SD
Moisture (g/100g raw weight)	55.2±1.7
Protein (g/ 100g raw weight)	45.6±3.7
Fat (g/100g raw weight)	3.9±1.6
Shear stress (N/mm^2 raw epimysium)	38.4±6.8

N=18.

contained about 55±2% moisture. Raw epimysium samples had shear stress values of 38±7 N/mm^2. The main effects of treatments and their interactions were reported for each acid and alkali, separately. It was observed that, main effects were not significant for any of the parameters studied after HCl treatments (Table 5.2 and Table 5.3) but one or many of the two-way and three-way interactions were significant for all the parameters measured. Similarly, for CH_3COOH treated epimysium, main effects and either two-way or three-way interactions were provided when significant interactions were noted.

5.4.1.1 Weight gain

Collagen was known to absorb water when placed in an aqueous medium. In this experiment, weight gain by the epimysium reflected the net weight of liquid absorbed (degree of hydration) and protein released. Weight gain was computed as the *{(Wf–Wo / Wo)* 100}* where, *Wo* was the raw weight of epimysium; *Wf* was the weight after heating. Epimysial weight gain after HCl treatments (Table 5.2) was influenced by the pre-equilibration time ($P<0.0001$), concentration ($P<0.0001$) and the following interactions, pre-equilibration*concentration ($P<0.0001$), temperature*concentration ($P<0.001$) and temperature*concentration*pre-equilibration ($P<0.05$) (Table 5.2). As such, both main effects and interaction means are presented (Table 5.3 and Table 5.4).

Table 5.2 Probabilities of main effects of treatments and their two-way and three-way interactions for physicochemical properties of HCl treated epimysium

		Physicochemical Property				
	df	Weight gain (%)	Thickness change (%)	Protein release (%)	OPA sensitive free amino groups (glycine mg/g raw epimysium)	[1]Shear stress (N/mm^2)
Block	5	P<0.018	P<0.002	P<0.352	P<0.394	P<0.077
Temperature	1	P<0.235	P<0.063	P<0.003	P<0.016	P<0.006
Block*Temperature	5	P<0.237	P<0.814	P<0.0001	P<0.0001	P<0.0001
Pre-equilibration	2	P<0.0001	P<0.001	P<0.011	P<0.0001	P<0.0001
Temperature*Pre-equilibration	2	P<0.449	P<0.113	P<0.088	P<0.123	P<0.001
Concentration	2	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Temperature*Concentration	2	P<0.001	P<0.0001	P<0.0001	P<0.0001	P<0.255
Pre-equilibration* Concentration	4	P<0.0001	P<0.0001	P<0.361	P<0.305	P<0.588
Temperature*Pre-equilibration* Concentration	4	P<0.0435	P<0.003	P<0.468	P<0.888	P<0.938

[1]Maximum shear force acting on a unit cross sectional area was corrected for thickness of raw epimysium (mm).

Table 5.3 Main effects of HCl concentration, pre-equilibration time and temperature on physicochemical properties of cow epimysium

		Physicochemical property				
		**Weight gain (%)	**Thickness change (%)	*Protein release (%)	*OPA sensitive free amino groups (glycine mg/g raw epimysium)	*Shear stress (N/mm^2)
Concentration	0.1M	124.4±41.4	168.6±70.3	1.3±1.0	1.1±0.5	8.2±5.7
	0.25M	74.4±26.0	149.0±53.8	2.2±1.6	1.3±0.6	5.5±4.8
	0.5M	36.0±21.2	98.5±64.3	5.7±5.6	1.9±0.9	3.3±3.9
	P	0.0001	0.0001	0.0001	0.0001	0.0001
	LSD	8.16	19.0	0.72	0.19	0.94
Pre-equilibration	0 min	56.8±26.0	118.6±39.6	2.4±2.7	1.2±0.6	8.3±6.5
	90 min	80.3±45.1	138.3±65.5	3.4±4.5	1.5±0.8	4.7±4.2
	180 min	97.6±57.5	159.2±89.3	3.4±4.1	1.6±0.8	4.0±3.6
	P	0.0001	0.001	0.011	0.0001	0.0001
	LSD	8.16	19.0	0.72	0.19	4.73
Temperature	55 ºC	80.9±39.3	132.5±44.1	1.0±0.3	1.1±0.5	8.3±5.7
	70 ºC	75.6±54.6	144.9±87.6	5.1±4.6	1.8±0.8	3.0±2.9
	P	0.235 (NS)	0.063 (NS)	0.003	0.016	0.006
	LSD	-	-	2.04	0.51	3.04

Means ± SD of parameters are shown above. ** Symbol indicates significant three-way interactions. * Symbol indicates significant two-way interactions.
NS= Not significant

Accordingly, weight gained by epimysium was significantly reduced with the increasing concentration of HCl within all pre-equilibration treatments and also subsequent to heating at 55 ºC and 70 ºC (Table 5.4). After pre-equilibrating epimysium samples in 0.1 M HCl for 180 min and subsequent heating to 70 ºC, weight gains were considerably higher than for other treatments. This phenomenon must have contributed to the observed three-way interaction among temperature*concentration*pre-equilibration. The two-way interaction of temperature*concentration was evident as weight gain was lowest (22-24%) after heating to 70 ºC in 0.5 M HCl while weight gain at the other HCl concentrations at 55 and 70 ºC were distributed in the ranges of 39-147% and 60-180%, respectively. In other words, at very high concentrations of HCl at 70 ºC, the ability of epimysium to hold water was reduced. This may be resulting from the release of epimysial proteins. At 55 ºC, epimysium samples directly heated with HCl (0 min pre-equilibration) had gained more weight than epimysium samples heated in water indicating the ability of collagen to hold more liquid at low pH values (Table 5.5). At 70 ºC, epimysium heated with 0.1 M and 0.25 M HCl (0 min pre-equilibration) had gained more weight than those heated in water. At 70 ºC, epimysium samples treated with 0.5 M HCl (with no pre-equilibration treatment) and those heated in water had similar weight gain data (Table 5.5) even though physicochemical properties of epimysium were likely to be different after acid and water treatments.

Table 5.4 Three-way interaction means of physicochemical properties of epimysium treated with HCl.

		55 ºC			70 ºC		
		Pre-equilibration			Pre-equilibration		
	HCl	No Equilibration	90 min	180 min	No Equilibration	90 min	180 min
Weight gain (%)	0.1M	86.6±16.4	130.0±41.6	147.0±20.5	79.2±6.3	123.96±14.9	179.9±28.3
	0.25M	54.9±7.6	73.8±18.4	88.9±11.2	59.5±19.9	80.0±33.2	89.0±38.1
	0.5M	38.8±15.1	52.0±10.9	56.4±12.0	22.1±15.4	22.4±21.7	24.6±24.3
	P[1]	0.0001	0.001	0.0001	0.0001	0.0001	0.0001
	LSD[2]	10.7	32.8	12.8	13.4	31.9	30.6
Thickness change (%)	0.1M	92.6±27.9	130.3±39.1	169.3±45.4	152.6±51.3	200.0±25.7	266.7±72.3
	0.25M	107.4±45.1	136.5±38.3	176.7±25.5	123.3±10.5	175.5±74.0	174.8±70.3
	0.5M	104.9±12.3	134.1±41.7	140.6±50.4	130.9±49.6	53.4±24.6	26.9±6.0
	P[1]	0.653 (NS)	0.947 (NS)	0.066 (NS)	0.398 (NS)	0.003	0.0001
	LSD[2]	-	-	-	-	76.2	75.8

Mean ± SD of only the properties with a significant interaction for 'Temperature*Pre-equilibration*Concentration' are shown above.
P[1] and LSD[2] compare only the means across different concentration treatments.
NS= Not significant

Table 5.5 T-test comparison of physicochemical properties of epimysium treated in acid and water without a pre-equilibration treatment.

		Physicochemical property					
Acid (0 min pre-equilibration)	Temperature	Concentration	Weight gain (%)	Thickness change (%)	Shear stress (N/mm^2)	Protein released (g/100g raw epimysium)	OPA sensitive free amino groups (glycine mg/g raw epimysium)
HCl	55 °C	0.1 M	86.56±10.20	92.56±27.95	15.61±7.40	0.66±0.13	0.94±0.55
		P>|t|	0.0001	0.001	0.066	0.820	0.780
		0.25 M	54.90±7.59	107.35±45.10	11.62±7.30	0.78±0.20	0.95±0.52
		P>|t|	0.0001	0.001	0.023	0.274	0.738
		0.5M	38.79±15.14	104.88±12.29	9.16±4.88	0.98±0.27	0.86±0.37
		P>|t|	0.005	0.0001	0.008	0.05	0.983
		Water	**12.32±6.14**	**25.55±9.47**	**28.95±7.78**	**0.63±0.27**	**0.86±0.45**
	70 °C	0.1 M	79.18±3.95	152.64±51.28	6.49±3.36	1.51±0.93	0.84±0.37
		P>|t|	0.0001	0.081	0.744	0.038	0.933
		0.25 M	59.51±19.95	123.29±10.52	4.53±2.35	2.65±1.08	1.20±0.22
		P>|t|	0.017	0.218	0.183	0.001	0.147
		0.5M	22.13±15.41	130.98±49.61	2.35±1.45	7.90±1.62	2.17±0.46
		P>|t|	0.184	0.285	0.018	0.001	0.001
		Water	**33.08±6.75**	**102.17±38.0**	**7.20±3.93**	**0.59±0.12**	**0.81±0.55**
CH_3COOH	55 °C	0.1 M	55.57±7.33	59.61±19.43	14.55±11.44	0.65±0.23	0.44±0.26
		P>|t|	0.0001	0.009	0.449	0.893	0.318
		0.25 M	68.06±16.91	59.84±20.51	13.46±12.25	0.64±0.28	0.49±0.25
		P>|t|	0.0001	0.011	0.388	0.953	0.480
		0.5 M	80.60±14.62	98.60±22.89	10.88±4.63	0.77±0.24	0.44±0.27
		P>|t|	0.0001	0.0001	0.226	0.277	0.339
		Water	**10.37±3.50**	**27.99±14.47**	**20.69±15.32**	**0.63±0.18**	**0.58±0.20**

The null hypothesis $\mu_{water} = \mu_{acid/alkali}$ is rejected when P>|t| is greater than 0.05, N=6 for acids.

Table 5.5 continued……..

Acid (0 min pre-equilibration)	Temperature	Physicochemical property: Concentration	Weight gain (%)	Thickness change (%)	Shear stress (N/mm^2)	Protein released (g/100g raw epimysium)	OPA sensitive free amino groups (glycine mg/g raw epimysium)
CH_3COOH	70 °C	0.1 M	61.87±12.21	199.38±33.28	6.20±4.74	1.07±0.46	0.78±0.55
		P>|t|	0.014	0.410	0.986	0.103	0.21
		0.25 M	75.58±28.95	147.50±39.07	5.93±4.85	1.14±0.58	0.53±0.28
		P>|t|	0.025	0.036	0.910	0.105	0.572
		0.5 M	89.69±15.03	170.53±39.32	5.89±4.39	1.16±0.31	0.62±0.40
		P>|t|	0.0001	0.0036	0.895	0.016	0.372
		Water	**42.30±10.58**	**106.89±12.60**	**6.24±4.49**	**0.68±0.28**	**0.44±0.026**

The null hypothesis $\mu_{water} = \mu_{acid/alkali}$ is rejected when P>|t| is greater than 0.05, N=6 for acids.

CH_3COOH treated epimysium had gained more weight as concentration of acid was increased ($P<0.0001$) and also as pre-equilibration time increased `($P<0.0001$) (Table 5.6 and Table 5.7). Temperature increase from 55 to 70 ºC had no effect on the weight gain by CH_3COOH treated epimysium. Epimysium heated with any of the 3 concentrations of CH_3COOH had gained more weight than epimysium heated in water at both 55 and 70 ºC (Table 5.5). Thus, it was clear that low pH of CH_3COOH improved the liquid holding ability of epimysial collagen.

5.4.1.2 Thickness change

Thickness of raw epimysium and its variation among animals used in this study were presented in Table 5.8. Accordingly, thickness of epimysium pieces used in HCl and CH_3COOH experiments was 1.99-3.46 mm and 1.83-3.44 mm, respectively. Characteristically, thickness of collagen and collagen dominant epimysium were increased as a result of heating. The percentage thickness change was computed as *{(Tf-To / To)*100}*, where *To* was the initial thickness of the epimysium piece and *Tf* was the thickness after treatments. Thickness change of HCl treated epimysium was influenced by pre-equilibration time ($P<0.001$), concentration ($P<0.0001$) and also by two-way and three-way interactions as follows, pre-equilibration*concentration ($P<0.0001$), temperature*concentration ($P<0.0001$) and temperature*concentration*pre-equilibration ($P<0.05$) (Table 5.2). Therefore, main effects and the interaction means were presented (Table 5.3 and Table 5.4).

As a whole, increasing concentration of HCl from 0.1 to 0.5 M within any of the pre-equilibration treatments studied did not change the epimysial thickness change at 55 ºC (Table 5.4). Similarly at 70 ºC, increasing concentration of HCl did not change the epimysial thickness change at 0 min (pre-equilibration treatment); however, increasing concentration of HCl within 90 and 180 min pre-equilibration treatments and also subsequent heating at 70 ºC had

Table 5.6 Probabilities of main effects and their two-way and three-way interactions of physicochemical properties of epimysium following CH_3COOH treatment

		Physicochemical Property				
	df	Weight gain (%)	Thickness change (%)	Protein release (%)	OPA sensitive free amino groups (glycine mg/g raw epimysium)	[1]Shear stress (N/mm^2)
Block	5	P<0.070	P<0.0001	P<0.006	P<0.008	P<0.023
Temperature	1	P<0.508	P<0.0001	P<0.007	P<0.036	P<0.060
Block*Temperature	5	P<0.353	P<0.071	P<0.057	P<0.01	P<0.0001
Pre-equilibration	2	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Temperature*Pre-equilibration	2	P<0.912	P<0.400	P<0.900	P<0.7715	P<0.001
Concentration	2	P<0.0001	P<0.0001	P<0.087	P<0.646	P<0.001
Temperature*Concentration	2	P<0.302	P<0.693	P<0.391	P<0.231	P<0.170
Pre-equilibration* Concentration	4	P<0.427	P<0.852	P<0.522	P<0.207	P<0.900
Temperature*Pre-equilibration* Concentration	4	P<0.612	P<0.080	P<0.523	P<0.040	P<0.762

[1]Maximum shear force acted on unit cross sectional area was corrected for thickness of raw epimysium (mm).
Predetermined significance level of the experiment was P<0.05.

Table 5.7 Main effects of CH_3COOH concentration, pre-equilibration time and temperature on physicochemical properties of cow epimysium

		Physicochemical property				
		Weight gain (%)	Thickness change (%)	Protein release (%)	**OPA sensitive free amino groups (glycine mg/g raw epimysium)	*Shear stress (N/mm^2)
Concentration	0.1M	84.9±26.9	113.3±48.2	1.1±0.4	0.80±0.4	8.7±7.8
	0.25M	110.9±39.2	133.9±56.1	1.3±0.6	0.77±0.5	7.9±7.7
	0.5M	128.3±47.8	154.8±59.5	1.3±0.6	0.83±0.5	6.8±6.2
	P	0.0001	0.0001	0.087 (NS)	0.646 (NS)	0.0001
	LSD	12.7	13.7	-	-	0.93
Pre-equilibration	0 min	72.9±20.36	109.2±50.6	0.9±0.4	0.55±0.3	9.5±8.9
	90 min	117.3±40.1	139.4±53.3	1.3±0.6	0.88±0.5	7.3±6.4
	180 min	134.1±38.8	153.4±61.2	1.5±0.7	0.97±0.5	6.6±6.1
	P	0.0001	0.0001	0.0001	0.0001	0.0001
	LSD	12.7	13.7	0.16	0.11	0.93
Temperature	55 ºC	106.1±45.9	102.9±45.4	1.0±0.5	0.68±0.4	10.2±8.9
	70 ºC	110.0±39.1	165.2±50.0	1.5±0.7	0.92±0.5	5.4±4.1
	P	0.508 (NS)	0.0001	0.007	0.036	0.06 (NS)
	LSD	-	21.2	0.26	0.21	-

Means ± SD of parameters with significant main effects are shown above. NS= Not Significant.
** Symbol indicates significant three-way interactions. * Symbol indicates significant two-way interactions.

Table 5.8 Thickness of raw **e**pimysium as influenced by animals

Thickness (mm)			
Animal	Used for HCl treatment	Animal	Used for CH_3COOH treatment
1	2.92±0.48	7	2.92±0.4
2	2.50±0.51	8	2.65±0.4
3	2.95±0.48	9	3.06±0.4
4	3.00±0.46	10	2.40±0.5
5	2.63±0.31	11	2.21±0.3
6	2.53±0.43	12	2.29±0.3

Mean ±SD. These epimysium pieces under each treatment were excised from random locations on the whole epimysium.

significantly reduced the thickness change of epimysium. For example, 90 min ($P<0.05$) and 180 min ($P<0.001$) pre-equilibration in 0.5 M HCl had diminished the thickness increase. Epimysium samples equilibrated for 180 min with 0.5 M HCl were similar in texture to thickened glue, such that pieces could not be handled. The observed interaction among temperature*pre-equilibration*concentration, might be a result of aforementioned, not-systematic thickness changes of epimysium. Thickness change of HCl treated epimysium was increased more than those heated with water at 55 ºC (Table 5.5). Epmysium samples heated in water and HCl had shown similar thickness changes at 70 ºC (Table 5.5).

With the increasing concentration of CH_3COOH ($P<0.0001$), pre-equilibration time in acid ($P<0.0001$) and the temperature of heating ($P<0.0001$), thickness change of epimysium samples had increased significantly (Table 5.6 and Table 5.7). Epimysium samples heated in CH_3COOH (with no pre-equilibration time) had higher thickness changes than those heated in water at both 55 and 70 ºC (Table 5.5).

5.4.1.3 Protein release

Epmysial protein release after heating with acid was significantly influenced by temperature ($P<0.003$), pre-equilibration ($P<0.05$) and also by the interaction, temperature*concentration ($P<0.0001$) (Table 5.2). Therefore, main effects and two-way interaction means were presented (Table 5.3 and Table 5.9). Accordingly, this interaction might be resulting from the very high protein release (10±4.8% on a weight basis) after heating with 0.5 M HCl at 70 ºC (Table 5.9) while at all other combinations of temperature and concentration of HCl the amount of protein release was less than 3.5% (on a weight basis). Further, HCl treated epimysium at 55 ºC had released ≤1.2±0.4% protein (on raw weight basis). Long pre-equilibration treatments had increased protein release more than 0 min pre-equilibration treatment in acid ($P<0.05$; LSD=0.71); for example, about 3.4% proteins (on raw weight basis) were liberated after 90 and 180 min pre-equilibration and only about 2.4% proteins were liberated at 0 min pre-equilibration treatment. The amounts of protein liberated from epimysium after heating with 0.1, 0.25 and 0.5 M HCl (with no pre-equilibration treatments) at 55 ºC were similar to the amounts of protein released after heating with water (Table 5.5). But raising the temperature to 70 ºC had favoured release of more proteins with HCl than with water at the same temperature (Table 5.5).

Subsequent to CH_3COOH treatment, epimysial protein release was increased with the length of the pre-equilibration treatment ($P<0.0001$) and also with the increasing temperature from 55 to 70 ºC ($P<0.05$) (Table 5.6 and Table 5.7). The increasing concentration of CH_3COOH had no effect in increasing the protein release. The maximum amount of protein release after heating with CH_3COOH (any combination of concentration and pre-equilibration time) at 70 ºC (<2%) (Table 5.7) was about 5 times less than the maximum amount of protein released (~10%) with 0.5 M HCl at 70 ºC (Table 5.9). Epimysium samples heated with 0.5 M CH_3COOH at 70 ºC (with no-pre-equilibration treatment) had released more protein than those heated with water

Table 5.9 Two-way interaction means (least square means) for HCl treated epimysium

	Interaction		Interaction mean ± SD
	Temperature	Concentration	
Protein released	55 ºC	0.1 M	0.82±0.2
(g/100 g raw epimysium)		0.25 M	0.91±0.3
		0.5 M	1.22±0.4
	70 ºC	0.1 M	1.86±1.2
		0.25 M	3.40±1.5
		0.5 M	10.10±4.6
OPA reactive amino groups	55 ºC	0.1 M	1.04±0.5
(glycine mg/g raw epimysium)		0.25 M	1.03±0.5
		0.5 M	1.12±0.5
	70 ºC	0.1 M	1.18±0.5
		0.25 M	1.54±0.7
		0.5 M	2.59±0.8
	Temperature	Pre-equilibration	
Shear stress (N/mm^2)	55 ºC	0 min	12.13±6.8
		90 min	6.69±4.4
		180 min	6.19±3.8
	70 ºC	0 min	4.46±2.9
		90 min	2.68±2.1
		180 min	1.79±1.1

Mean ±SD for epimysial properties with significant two-way interaction, Temperature * Concentration, are given.

($P<0.05$) (Table 5.5). All other concentrations of CH_3COOH at 55 and 70 ºC were similar to water treatment in their ability to liberate protein (Table 5.5).

Protein released from epimysium after acid treatment was presented as a percentage of total protein (Table 5.10) and values were computed as [*(Pr / Pt)*100*], where *Pr* was protein released after each treatment (measured as BCA assay sensitive groups) and *Pt* was total protein content of epimysium from each animal (measured as Kjeldahl nitrogen). Protein release data from all three pre-equilibration treatments within a concentration of acid treatment were pooled in order to calculate the above. Accordingly, epimysium had liberated similar amounts of proteins after both HCl and CH_3COOH treatments at 55 ºC. HCl had a prominent effect in liberating epimysial proteins at 70 ºC but CH_3COOH did not increase protein release under similar conditions of heating. For example, about 23±9.7% epimysial proteins (on a total protein basis) were released with 0.5 M HCl whereas, only about 3.4±1.4% proteins were released with 0.5 M CH_3COOH at 70 ºC.

Proteins released as a result of heating epimysium in HCl consisted of peptides of varying molecular weights. Those peptides were elucidated by SDS gel electrophoresis. Two peptide bands were identified as originating from collagen from samples previously heated at 55 ºC with 0.1 M HCl; (a) double strands of collagen (2α chains) which may be linked either as α1-α1 or as α1-α2 with an average molecular weight >200 kDa and (b) single helices separated from triple helices to produce α1 and α2 chains with molecular weights around 116 kDa (lane 1 of Figure 5.1). Also, there were other bands of unknown origin. Increasing concentration of HCl at 55 ºC had a clear effect on hydrolysis of epimysial proteins. For example, after heating in 0.25 and 0.5 M HCl, bands for double helices did not appear and the bands for single helices gradually became thinner (lane 2 and 3 of Figure 5.1). It is evident that peptides of random chain lengths

Table 5.10 Total proteins released from epimysium after acid treatments

Acid	Concentration	Protein released (%)	
		55 °C	70 °C
HCl	0.1 M	1.86±0.4	4.08±2.3
	0.25 M	2.06±0.6	9.64±7.2
	0.5 M	2.75±0.8	23.29±9.7
CH_3COOH	0.1 M	2.15±0.9	2.90±0.9
	0.25 M	2.28±1.1	3.15±1.3
	0.5 M	2.32±1.2	3.41±1.4

Percentage protein released was computed as *[(Pr / Pt)*100]* where *Pr* protein released after each concentration treatment irrespective of pre-equilibration treatment (BCA assay) and *Pt* is total protein content (Kjeldahl). Mean±SD, N=9.

were formed with all concentration of HCl at 70º C to develop a smear (lane 6, 7 and 8 of Figure 5.1). Epimysial proteins liberated after heating in CH_3COOH at 55 and 70 ºC had contained single and double strands of collagen (Figure 5.2). However, with 0.5 M CH_3COOH, thickness of protein bands were decreased indicating further hydrolysis of single and double strands of collagen.

5.4.1.4 O-Phthaldialdehyde (OPA) reactive free amino groups

As a result of combined acid and heat treatments, some of the proteins of epimysium were expected to hydrolyse to produce polypeptides of various chain lengths and also free amino acids. Free α and ε amino groups, thus produced were reacted with OPA. The release of free amino groups after HCl treatment was influenced by temperature ($P<0.0001$), pre-equilibration time ($P<0.0001$) and also by the interaction, temperature*concentration ($P<0.0001$) (Table 5.2). The main effects and interaction means for temperature*concentration were presented (Table 5.3 and Table 5.9). This interaction might be resulting from the higher release of OPA reactive

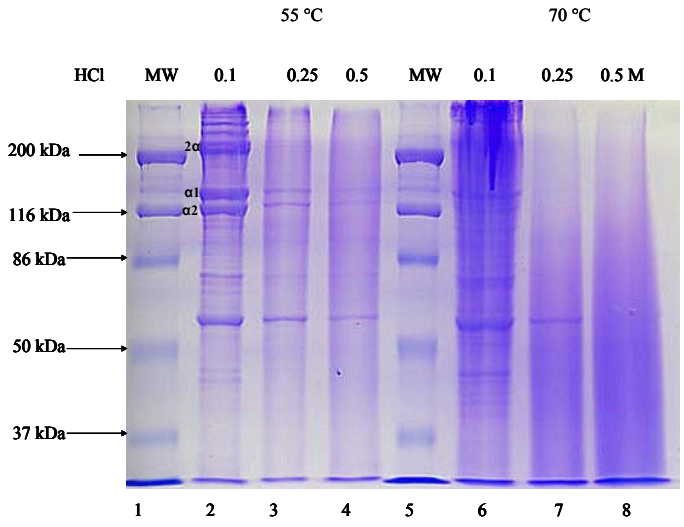


Figure 5.1 HCl derived peptides of epimysial collagen resolved on 8% SDS gels. Proteins were obtained after 180 min pre-equilibration in HCl and subsequent heating to 55 and 70 °C for 15 min. Each well was loaded with 3 µg of protein. Lane 1 and 5 shows molecular weight markers. Lanes 2 to 4- protein release after heating at 55 ˚C. Lane 6 to 8- protein release after heating at 70 ˚C. Single strands of collagen are α1 and α2. Double strand of collagen is 2α.

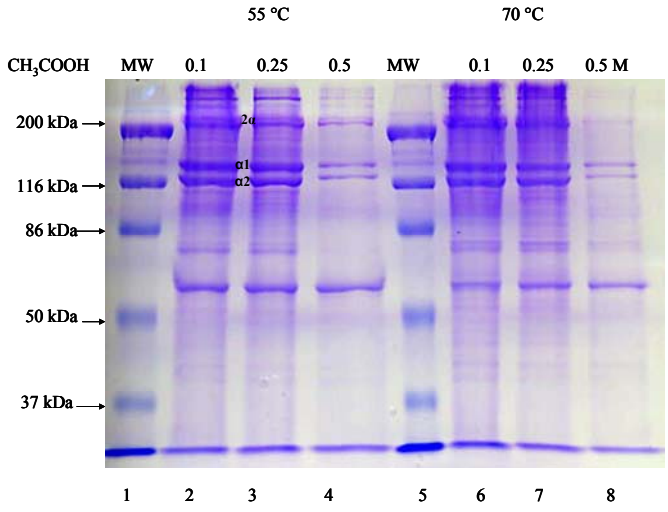


Figure 5.2 CH_3COOH derived peptides of epimysial collagen resolved on 8% SDS gels. Proteins were obtained after 180 min pre-equilibration in CH_3COOH and subsequent heating to 55 and 70 °C for 15 min. Each well was loaded with 3 µg of protein. Lane 1 and 5 shows molecular weight markers. Lanes 2 to 4- protein release after heating at 55 ˚C. Lane 6 to 8- protein release after heating at 70 ˚C. Single strands of collagen are α1 and α2. Double strand of collagen is 2 α.

amino groups with the increasing concentration of HCl at 70ºC while no similar increase was noted at 55 ºC (Table 5.9). Those epimysium samples that received long pre-equilibration treatments in HCl had released more OPA reactive groups than samples that did not receive a pre-equilibration treatment ($P<0.0001$; LSD= 0.19). For example, after 90 and 180 min pre-equilibration treatments about 1.5-1.6% OPA reactive groups (mg glycine per 100g epimysium) were released and this was significantly higher than 1.1% OPA reactive groups released at 0 min pre-equilibration treatment. At 55 ºC, epimysium samples heated in water and those heated in HCl (with no pre-equilibration treatment) had released similar amounts of free amino groups (Table 5.5). The amount of free amino groups released after heating epimysium with 0.1 and 0.25 M HCl (with no pre-equilibration treatment) at 70 ºC was similar to the amounts released with water at the same temperature. However, the combined treatments of 0.5 M HCl and heating at 70 ºC had released significantly higher amounts of free amino groups than samples heated in water at the same temperature (Table 5.5).

The release of OPA reactive amino groups from CH_3COOH treated epimysium was influenced by temperature ($P<0.05$), pre-equilibration time ($P<0.0001$ and also by the interaction temperature*pre-equilibration*concentration ($P<0.05$) (Table 5.6) and therefore, main effects and significant interaction means were presented (Table 5.7 and Table 5.11). The release of higher amounts of OPA reactive amino groups after 90 min pre-equilibration in 0.5 M CH_3COOH and subsequent heating of epimysium to 70 ºC than other treatment combinations might be the reason for the temperature*pre-equilibration*concentration interaction. The maximum amount of OPA reactive free amino groups released after CH_3COOH treatment was about 1% (on raw weight basis and that was similar to the values observed after HCl treatment at 55 ºC (Table 5.9). The effect of three concentrations of CH_3COOH (without pre-equilibration) was similar to that of water in releasing free amino groups at both 55 and 70 ºC (Table 5.5).

Table 5.11 Three-way interaction means of physicochemical properties of epimysium treated with CH_3COOH.

		55 ºC			70 ºC		
		Pre-equilibration			Pre-equilibration		
	CH_3COOH	No Equilibration	90 min	180 min	No Equilibration	90 min	180 min
OPA sensitive free amino groups (glycine mg/g raw epimysium)	0.1M	0.43±0.26	0.77±0.34	0.97±0.41	0.78±0.31	0.83±0.30	1.04±0.33
	0.25M	0.49±0.25	0.77±0.40	0.76±0.27	0.53±0.28	0.93±0.43	1.18±0.81
	0.5M	0.44±0.27	0.73±0.43	0.80±0.45	0.62±0.40	1.30±0.66	1.07±0.41
	P[1]	0.746	0.937	0.206	0.095	0.069	0.738
	LSD[2]	-	-	-	-	-	-

Mean ±SD for epimysial properties with significant three-way interaction, Temperature*Pre-equilibration time* Concentration, are given.
P[1] and LSD[2] compare only the means across concentration treatments.

5.4.1.5 Shear stress

Shear stress was considered as a direct measurement of the structural strength of epimysium. In this study, shear stress was presented as the force applied on a unit cross-sectional area of epimysium (N/mm^2) after correcting for thickness differences in raw epimysium pieces. According to the statistical model used, shear stress after HCl treatment was influenced by the concentration ($P<0.0001$), pre-equilibration ($P<0.0001$), temperature ($P<0.05$) and also by the interaction temperature*pre-equilibration ($P<0.001$). Therefore, main effects and two-way interaction means were presented (Table 5.3 and Table 5.9).

The shear stress was decreased considerably from 0 to 90 min pre-equilibration and also from 90 to 180 min pre-equilibration in HCl and subsequent heating to 70 ºC (Table 5.9). On the other hand, shear stress after 0 min pre-equilibration in HCl and subsequent heating to 55 ºC was considerably higher than the other combinations of temperature and HCl concentration. It was thought that both situations explained above had contributed to the interaction temperature*pre-equilibration.

Each increment of HCl concentration had significantly decreased the shear stress ($P<0.0001$; LSD=4.73); for example, shear stress values after 0.1, 0.25 and 0.5 M HCl treatments were 41.1, 27.5, and 16.3 N/mm^2, respectively. At the low temperature treatment (55 ºC), shear stress of HCl treated samples (without a pre-equilibration treatment) were significantly lower than those heated in water. However, as the temperature was increased to 70 ºC, the effect of acid was eliminated except at 0.5 M HCl; thus, epimysium heated in HCl and water had similar shear stress values (Table 5.5).

The raw epimysium had an average shear force value of about 38±7 N/mm^2 (Table 5.1). A three-fold reduction in shear stress (12±7 N/mm^2) was observed after heating to 55 ºC with HCl

(at 0 min pre-equilibration). Pre-equilibration for 90 min with HCl and subsequent heating to 70 °C had reduced the shear stress by about twelve times (2.7±2 N/mm^2) (Table 5.9).

Shear stress of CH_3COOH treated epimysium was influenced by concentration ($P<0.001$), pre-equilibration time ($P<0.0001$) and also by the interaction temperature*pre-equilibration ($P<0.001$) (Table 5.6). Main effects and the two-way interaction means for temperature*pre-equilibration were provided (Table 5.7 and Table 5.12). Shear stress values obtained after 0 min pre-equilibration in CH_3COOH and subsequent heating to 55 °C were considerably higher than that obtained after other combinations of temperature and pre-equilibration time treatments. This might have driven the interaction temperature*pre-equilibration. Epimysium heated in both CH_3COOH and water had similar shear stress values (Table 5.5).

Table 5.12 Two-way interaction means (least square means) of physicochemical properties of epimysium treated with CH_3COOH

	Interaction		Interaction mean
	Temperature	Pre-equilibration	
Shear stress (N/mm^2)			
	55 ºC	0 min	12.96±10.9
		90 min	9.32±7.7
		180 min	8.28±7.5
	70 ºC	0 min	6.01±4.3
		90 min	5.26±4.1
		180 min	4.98±3.9

Mean ±SD of epimysial properties with significant two-way interaction, Temperature*Pre-equilibration time, are given.

5.4.2 Results of alkali treatments

During a preliminary experiment, it was observed that a pre-equilibration treatment of 120 min in 0.1 M NaOH and subsequent heating at 70 ºC for 15 min could completely solubilize cow epimysium. Because the objective of the experiment was to decrease shear stress without fully converting epimysial collagen to gelatine, it was decided to use fairly low concentrations of NaOH (0.01, 0.025 and 0.05 M). Based on the experimental model used, split-plot, the probabilities of main effects and interactions for physicochemical properties of alkali treated epimysium were summarized in Table 5.13 (NaOH) and Table 5.15 (NH_4OH). Wherever 2-way and 3-way interactions were observed significant, interaction means were provided.

5.4.2.1 Weight gain

Epimysial weight gain after NaOH treatments was influenced by temperature ($P<0.05$), pre-equilibration time ($P<0.001$) and concentration ($P<0.0001$). Weight gain was also influenced by the interaction pre-equilibration*concentration ($P<0.0001$) (Table 5.13). Two-way interaction means for pre-equilibration*concentration were given in Table 5.14. After 90 and 180 min pre-equilibration treatment in 0.05 M NaOH epimysial weight gain was increased to 61.8±14.7% and 73.7±7.7%, respectively. These weight gains were significantly higher than the weight gain values reported for the other combinations of concentration and pre-equilibration treatments. High values for weight gain observed after 90 and 180 min pre-equilibration in 0.05 M NaOH might have contributed to the observed interaction equilibration*concentration. A significant increase in epimysial weight was noted after the temperature increase from 55 ºC (~36%) to 70 ºC (~48%) ($P<0.05$; LSD=10.6).

Table 5.13 Probabilities of main effects and their two-way and three-way interactions of physicochemical properties of epimysium following NaOH treatment

		Physicochemical property				
	df	Weight gain (%)	Thickness change (%)	Protein release (%)	OPA sensitive free amino groups (glycine mg/g raw epimysium)	[1]Shear stress (N/mm^2)
Block	2	P<0.266	P<0.526	P<0.111	P<0.2571	P<0.296
Temperature	1	P<0.042	P<0.671	P<0.117	P<0.0326	P<0.016
Block*Temperature	2	P<0.357	P<0.023	P<0.069	P<0.8367	P<0.014
Pre-equilibration	2	P<0.001	P<0.678	P<0.0001	P<0.0002	P<0.733
Temperature*Pre-equilibration	2	P<0.439	P<0.442	P<0.110	P<0.2134	P<0.584
Concentration	2	P<0.0001	P<0.0001	P<0.0001	P<0.0042	P<0.003
Temperature*Concentration	2	P<0.165	P<0.782	P<0.242	P<0.4627	P<0.021
Pre-equilibration* Concentration	4	P<0.0001	P<0.074	P<0.086	P<0.3365	P<0.149
Temperature*Pre-equilibration* Concentration	4	P<0.315	P<0.935	P<0.089	P<0.5442	P<0.179

[1]Maximum force applied on a unit area of epimysium was corrected for the raw epimysium thickness (mm).

Table 5.14 Two-way interaction means (least square means) of physicochemical properties of epimysium treated with NaOH

	Interaction		Interaction means
	Pre-equilibration	Concentration	
Weight gain (%)			
	0 min	0.01 M	29.2±14.4
		0.025 M	34.8±5.8
		0.05 M	40.6±9.1
	90 min	0.01 M	32.9±14.8
		0.025 M	37.3±6.9
		0.05 M	61.8±14.7
	180 min	0.01 M	23.3±14.4
		0.025 M	43.1±8.4
		0.05 M	73.7±7.7
	Temperature	Concentration	
Shear stress (N/mm^2)			
	55 ºC	0.01 M	29.1±11.3
		0.025 M	19.5±7.5
		0.05 M	4.1±5.9
	70 ºC	0.01 M	4.1±1.4
		0.025 M	4.1±1.4
		0.05 M	2.9±1.5

Means±SD of epimysial properties with significant two-way interaction, Pre-equilibration*Concentration are given.

NH_4OH concentration ($P<0.001$), pre-equilibration treatment ($P<0.0001$) and the temperature of heating ($P<0.001$) had significantly influenced the epimysial weight gain (Table 5.15). Therefore, the means of main effects were given (Table 5.16). In view of that, epimysial weight gain was significantly increased, as concentration of NH_4OH was increased from 0.1 M to 0.25 M but further increase in concentration, to 0.5 M, did not increase weight gain. Increasing pre-equilibration time from 0 min to 90 min had increased weight gain significantly but further increase, to 180 min, had no effect on weight gain. Heating epimysium at 70 ºC had favoured higher epimysial weight gains than at 55 ºC (Table 5.16).

At 55 ºC, all concentrations of NaOH and NH_4OH had increased epimysial water uptake and thereby increased the weight gain more than samples heated in water (Table 5.17). As the temperature was increased, the effect of alkali was eliminated and consequently alkali treated samples had similar weights to those heated in water (Table 5.17).

5.4.2.2. Thickness change

The raw thickness of epimysium pieces used in NaOH and NH_4OH experiments were in the range of 2.19-3.22 mm and 1.91- 2.92 mm (Table 5.18). Thickness of NaOH treated epimysium was influenced by the concentration of NaOH ($P<0.0001$) (Table 5.13). Accordingly, each incremental change in NaOH concentration increased the thickness of epimysium significantly (Table 5.19), and the highest thickness increase was achieved at the 0.05 M NaOH concentration (~87.3±43.4%).

The thickness of NH_4OH treated epimysium was influenced by temperature of heating ($P<0.05$) and the interaction temperature*pre-equilibration time ($P<0.05$) (Table 5.15) and thus, main effects and interaction means were given (Table 5.16 and Table 5.20).

Table 5.15 Probabilities of main effects and their two-way and three-way interactions of physicochemical properties of epimysium following NH_4OH treatment

		Physicochemical property			
	df	Weight gain (%)	Thickness change (%)	Protein released (%)	[1]Shear stress (N/mm^2)
Block	2	P<0.021	P<0.526	P<0.350	P<0.134
Temperature	1	P<0.001	P<0.016	P<0.147	P<0.001
Block*Temperature	2	P<0.969	P<0.0001	P<0.200	P<0.239
Pre-equilibration	2	P<0.0001	P<0.092	P<0.0001	P<0.837
Temperature*Pre-equilibration	2	P<0.864	P<0.041	P<0.195	P<0.684
Concentration	2	P<0.001	P<0.735	P<0.0001	P<0.145
Temperature*Concentration	2	P<0.809	P<0.486	P<0.067	P<0.909
Pre-equilibration* Concentration	4	P<0.405	P<0.653	P<0.117	P<0.677
Temperature*Pre-equilibration* Concentration	4	P<0.952	P<0.166	P<0.361	P<0.752

[1]Maximum force applied on a unit area of epimysium was corrected for the raw epimysium thickness (mm).

Table 5.16 Main effects of NH_4OH concentration, pre-equilibration and heating times on physicochemical properties of cow epimysium

		Physicochemical property			
		Weight gain (%)	*Thickness change (%)	Protein release (%)	Shear stress (N/mm^2)
Concentration	0.1M	36.0±11.4	59.5±53.6	1.2±0.3	22.7±17.7
	0.25M	43.5±15.2	61.9±52.5	1.5±0.6	22.3±17.8
	0.5M	46.7±12.9	62.4±49.6	1.8±0.6	20.9±17.6
	P	0.001	0.735 (NS)	0.05	0.145 (NS)
	LSD	4.89	-	0.24	-
Pre-equilibration	0 min	34.9±12.6	59.2±48.3	1.1±0.3	22.3±17.6
	90 min	43.7±12.8	58.1±47.0	1.6±0.4	21.9±17.4
	180 min	47.7±13.3	66.5±59.3	1.9±0.7	21.8±18.3
	P	0.0001	0.092 (NS)	0.0001	0.837 (NS)
	LSD	4.89	-	0.24	-
Temperature	55 ºC	32.2±8.0	13.7±7.3	1.4±0.5	38.9±3.5
	70 ºC	51.9±10.9	108.8±23.5	1.7±0.7	5.0±2.6
	P	0.001	0.016	0.091(NS)	0.001
	LSD	1.50	52.63	-	3.98

Means ± SD of parameters with significant main effects are shown above. NS= Not Significant.
* Symbol stands for significant two-way interactions

Table 5.17 T-test comparison of physicochemical properties of epimysium treated in alkali and water without a pre-equilibration treatment.

		Physicochemical property					
Alkali (No pre-equilibration)	Temperature	Concentration	Weight gain (%)	Thickness change (%)	Shear stress (N/mm^2)	Protein released (g/100g raw epimysium)	OPA sensitive free amino groups (glycine mg/g raw epimysium)
NaOH	55 °C	0.01 M	16.2±0.5	33.9±6.1	22.2±12.3	0.7±0.1	0.5±0.1
		P>|t|	0.002	0.141	0.944	0.474	0.625
		0.025 M	30.3±4.2	40.5±15.9	31.3±7.9	1.2±0.4	0.9±0.2
		P>|t|	0.001	0.158	0.477	0.544	0.089
		0.05M	36.3±11.4	54.7±7.8	20.3±9.9	1.4±0.7	0.9±0.6
		P>|t|	0.012	0.011	0.811	0.399	0.484
		Water	**7.1±2.2**	**21.7±9.8**	**23.1±16.2**	**0.9±0.5**	**0.8±0.2**
	70 °C	0.01 M	42.1±4.0	77.6±33.6	3.8±1.7	1.08±0.11	0.60±0.29
		P>|t|	0.794	0.759	0.589	0.818	0.134
		0.025 M	39.3±2.8	80.2±8.8	3.9±1.2	1.14±0.50	0.55±0.28
		Pr>|t|	0.809	0.765	0.587	0.986	0.097
		0.05 M	44.9±4.8	100.0±47.7	3.7±1.8	1.13±1.08	1.09±0.34
		P>|t|	0.489	0.702	0.563	0.997	0.722
		Water	**40.6±8.4**	**86.4±32.2**	**4.9±2.7**	**1.1±0.4**	**1.0±0.2**
NH_4OH	55 °C	0.1 M	22.2±2.3	12.4±6.1	39.4±2.9	0.9±0.2	ND
		P>|t|	0.042	0.221	0.198	0.613	
		0.25 M	24.7±1.8	18.3±3.5	39.3±1.9	1.0±0.5	ND
		P>|t|	0.014	0.373	0.197	0.413	
		0.5 M	28.1±4.4	11.7±4.2	38.8±5.3	1.2±0.1	ND
		P>|t|	0.016	0.199	0.224	0.034	
		Water	**14.68±3.76**	**30.33±20.54**	**24.11±16.86**	**0.75±0.23**	**ND**

The null hypothesis $\mu_{water} = \mu_{acid/alkali}$ is rejected when P>|t| is greater than 0.05. N=3 for alkalis. ND= not determined.

Table 5.17 continued……

		Physicochemical property					
Alkali (No pre-equilibration)	Temperature	Concentration	Weight gain (%)	Thickness change (%)	Shear stress (N/mm^2)	Protein released (g/100g raw epimysium)	OPA sensitive free amino groups (glycine mg/g raw epimysium)
NH_4OH	70 °C	0.1 M	41.9±6.1	101.9±19.2	5.8±3.0	1.0±0.4	ND
		P>|t|	0.331	0.114	0.661	0.558	
		0.25 M	46.9±14.4	102.7±5.1	5.5±3.3	1.2±0.3	ND
		P>|t|	0.774	0.077	0.612	0.303	
		0.5 M	45.4±12.2	108.5±31.2	5.0±2.6	1.4±0.2	ND
		P>|t|	0.650	0.249	0.495	0.043	
		Water	**50.1±11.2**	**140.7±27.3**	**7.3±4.7**	**0.9±0.2**	**ND**

The null hypothesis $\mu_{water} = \mu_{acid/alkali}$ is rejected when P>|t| is greater than 0.05. N=3 for alkalis. ND= not determined.

Table 5.18 Thickness of raw epimysium as influenced by animals

Thickness (mm)			
Animal	NaOH treatment	Animal	NH_4OH treatment
13	2.29±0.38	16	2.82±0.40
14	2.62±0.33	17	2.66±0.47
15	2.50±0.30	18	2.69±0.40

Mean ±SD. These epimysium pieces under each treatment were excised from random locations on the epimysium.

Table 5.19 Main effects of NaOH concentration, pre-equilibration and heating time on hysicochemical properties of cow epimysium

		Physicochemical properties				
		*Weight gain (%)	Thickness change (%)	Protein release (%)	OPA reactive free amino groups (glycine mg/g raw epimysium)	*Shear stress (N/mm^2)
Concentration	0.01M	28.4±14.3	48.8±30.3	1.0±0.3	0.8±0.3	16.1±14.6
	0.025M	38.4±7.6	67.7±33.5	1.7±0.9	1.2±0.6	16.5±13.8
	0.05M	58.6±17.5	87.4±43.4	2.0±0.9	1.3±0.6	11.2±9.4
	P	0.0001	0.0001	0.0001	0.05 (NS)	0.003
	LSD	10.59	16.11	0.31	-	3.20
Pre-equilibration	0 min	34.8±10.8	64.5±32.1	1.1±0.6	0.8±0.4	14.2±12.8
	90 min	44.0±17.7	67.8±32.4	1.7±0.9	1.3±0.6	14.3±12.9
	180 min	46.7±23.5	71.5±51.0	1.9±0.9	1.4±0.5	15.4±13.6
	P	0.0001	0.678 (NS)	0.0001	0.001	0.733
	LSD	5.96		0.31	0.29	
Temperature	55 ºC	36.0±20.1	43.6±22.5	1.2±0.6	1.0±0.4	25.6±9.3
	70 ºC	47.1±15.0	92.3±36.6	1.9±1.0	1.3±0.7	3.7±1.5
	P	0.042	0.671 (NS)	0.117 (NS)	0.05	0.016
	LSD	10.59	-	-	0.21	12.2

Means ± SD of parameters with significant main effects are shown above. NS= Not Significant.
* Symbol indicates significant two-way interactions

Table 5.20 Two-way interaction means (least square means) of physicochemical properties of epimysium treated with NH_4OH

	Interaction		Interaction means
Thickness change (%)	Temperature	Pre-equilibration	
	55 ºC	0 min	14.1±5.2
		90 min	14.3±7.9
		180 min	12.8±9.0
	70 ºC	0 min	104.3±18.8
		90 min	101.9±17.6
		180 min	120.1±30.2

Mean±SD of only the physicochemical properties for which interaction temperature*pre-equilibration was found significant are listed above.

At 55 ºC, thickness of NH_4OH treated epimysium was hardly changed (<15%) with the pre-equilibration treatments but as the temperature was raised to 70 ºC, epimysial thickness was increased (Table 5.20). NH_4OH treated epimysium had gained the highest thickness (120±30%) after 180 min pre-equilibration and subsequent heating to 70 ºC and this was considerably higher than the values reported for other combinations of temperature and pre-equilibration treatments. This situation might have contributed to the observed interaction temperature*pre-equilibration time Neither NaOH nor NH_4OH (at 0 min pre-equilibration) was superior to water in increasing thickness of epimysium during heating at 55 and 70 ºC (Table 5.17).

5.4.2.3 Protein release

The concentration of NaOH ($P<0.0001$) and pre-equilibration time ($P<0.0001$) had significantly influenced protein liberation from the epimysium (Table 5.13). Increasing alkali concentration from 0.01 to 0.025 M and increasing pre-equilibration time from 0 to 90 min had significantly increased the epimysial protein release (Table 5.19). However, the amount of protein liberated was <2% of the (raw) weight of the epimysium. Interestingly, the temperature increase from 55 to 70 ºC had no effect on epimysial protein liberation (Table 5.13).

Release of protein from the NH_4OH treated epimysium was similar to that explained under NaOH treatment and significantly influenced by the concentration of alkali ($P<0.05$) and pre-equilibration time in alkali ($P<0.0001$) (Table 5.13). Each incremental concentration of NH_4OH and pre-equilibration time had significantly increased protein release (Table 5.16), however, the maximum amount released was <2% of the weight of the epimysium (on raw weight basis). Temperature of heating had no effect on protein release after NH_4OH treatment (Table 5.13). Under the conditions of this experiment, protein liberation ability of NaOH was similar to that of water regardless the temperature of heating (Table 5.17). More epimysial proteins were released after heating epimysium with 0.5 M NH_4OH than with water at 55 and 70 ºC. The other concentrations of NH_4OH (0.1 and 0.25 M) and water treatment had released similar amounts of proteins at 55 and 70 ºC.

The epimysial peptides liberated after heating in alkali were separated on SDS gels (Figure 5.3 and Figure 5.4). After a 180 min pre-equilibration treatment in NaOH, epimysium had liberated peptides of varying molecular weights. For example single strands (α chains) with molecular weights >116 kDa and double strands (2α chains) with molecular weights >200 kDa) were liberated after heating at 55 ºC. In addition, short chain peptides of unknown origin were also noted (Figure 5.3). As observed the thickness of protein bands of single and double strands were increased as NaOH concentration was increased from 0.01 to 0.025 M at 55 ºC and this complies with the chemical analysis data discussed above. As NaOH concentration was increased to 0.05 M, band thickness of single and double strands was reduced; this may be resulting from disintegration of single and double strands to short chain peptides and perhaps amino acids. Protein bands of single and double strands of collagen, released after 0.01 M NaOH treatment (with a 180 min pre-equilibration treatment) at 70 ºC were thicker than those released after 0.025 and 0.05 M NaOH treatments (Figure 5.3). The likely reason was

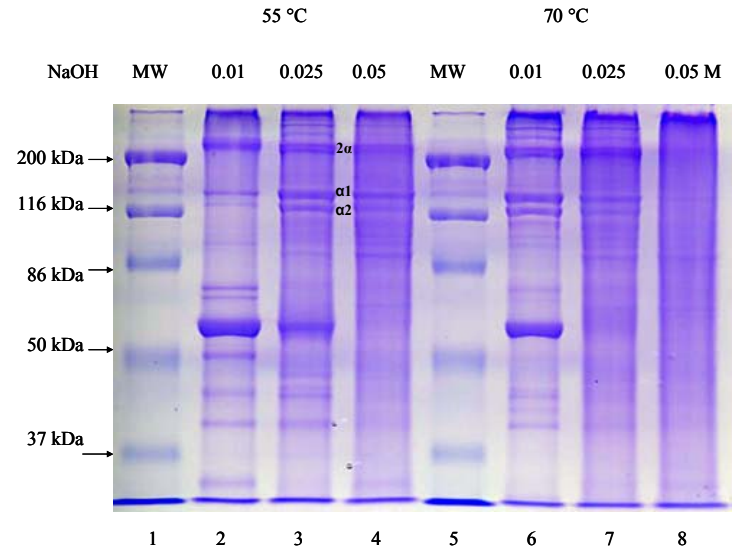
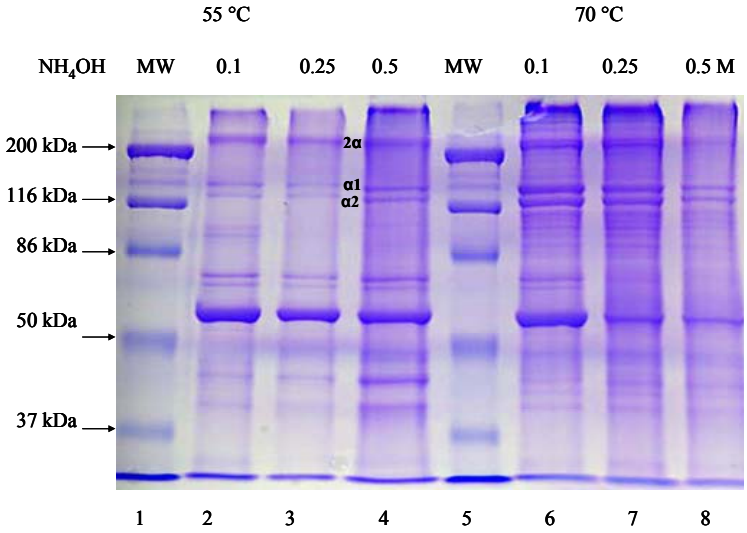


Figure 5.3. Peptides released from epimysial collagen subsequent to NaOH treatment and resolved on 8% SDS gel. Proteins were obtained after 180 min pre-equilibration of epimysium in NaOH and subsequent heating to 55 and 70 °C for 15 min. Each well was loaded with 3 µg of protein. Lane 1 & 5 –molecular weight markers. Lanes 2 to 4- protein bands after heating at 55 ˚C. Lane 6 to 8- protein bands after heating at 70 ˚C. α1 and α2 are single strands of collagen. 2α is a double strand of collagen.



5.4. Peptides released from epimysial collagen subsequent to NH_4OH treatment and resolved on 8% SDS gel. Proteins were obtained after 180 min pre-equilibration of epimysium in NH_4OH and subsequent heating to 55 and 70 °C for 15 min. Each well was loaded with 3 µg of protein. Lane 1 & 5 –molecular weight markers. Lanes 2 to 4- protein bands after heating at 55 ˚C. Lane 6 to 8- protein bands after heating at 70 ˚C. α1 and α2 are single strands of collagen. 2α is a double strand of collagen.

disintegration of single and double strands to produce short chain peptides due to the combined effect of high temperature (70 ºC) and alkali concentration. The protein bands, (single and double strands) emerging from 0.1 and 0.25 M NH_4OH treatments (with 180 min pre-equilibration) at 55 ºC (Figure 5.4) had similar thickness but as the concentration was increased to 0.05 M NH_4OH, a marginal thickness increase was noted. When the temperature was increased to 70 ºC, the thicknesses of the protein bands originating from 0.1 and 0.25 M NH4OH treatments were increased. However, as NH_4OH concentration was further increased to 0.5 M at 70 ºC, band thickness were reduced and this may be resulting from the disintegration of single and double strands to short peptides.

The total amounts of protein released after alkali treatments (BCA assay), after pooling data from pre-equilibration treatments, were presented as a percentage of total epimysial protein (Kjeldahl protein) (Table 5.21). Accordingly, subjected to the differences in concentrations, NaOH had a better ability to release epimysial proteins than NH_4OH.

Table 5.21 Effect of alkali concentration and temperature on epimysial protein release expressed on the basis of total epimysial proteins

		Protein released/ Total protein	
Acid/alkali	Concentration	55 °C	70 °C
NH_4OH	0.1 M	2.46±0.68	2.43±0.69
	0.25 M	2.62±0.79	3.73±1.50
	0.5 M	3.43±1.29	4.13±1.34
NaOH	0.01 M	1.87±0.56	2.39±0.71
	0.025 M	2.74±1.22	3.34±1.63
	0.05 M	3.81±2.45	4.02±2.86

Percentage protein released was computed as *[(Pr / Pt)*100]* where *Pr* protein released after each concentration treatment irrespective of pre-equilibration treatment (BCA assay) and *Pt* is total protein content (Kjeldahl). Mean±SD, N=9.

5.4.2.4 O-phthaldialdehyde (OPA) reactive free amino groups

Some degree of protein hydrolysis was expected as a result of NaOH treatment and thus OPA reactive free amino groups were measured in the alkali extracted epimysial proteins. The temperature of heating ($P<0.05$), alkali concentration ($P<0.05$) and the length of pre-equilibration treatment ($P<0.001$) had influenced the release of OPA reactive groups (Table 5.13). As the concentration was increased from 0.01 to 0.025 M NaOH and as the pre-equilibration time was increased from 0 to 90 min, more OPA reactive groups were liberated from epimysium (Table 5.19). Temperature increase from 55 to 70 ºC also had a similar positive effect on the release of OPA reactive groups. At 55 and 70 ºC, NaOH (without a pre-equilibration treatment) and water treatments had released similar amounts of OPA reactive amino groups (Table 5.17) and NaOH treatment was not superior to water treatment under the said experimental conditions.

The effect of NH_4OH on the release of OPA reactive groups was not studied as NH_3 and/or NH_4^+ interfered with the analytical method used and this is explained in section 5.5.2.

5.4.2.5 Shear stress

Epimysial shear stress after NaOH treatment was influenced by temperature ($P<0.05$), concentration ($P<0.05$) and also by the interaction temperature*concentration ($P<0.05$) (Table 5.13). Therefore, main effects and two-way interaction means were given (Table 5.14 and Table 5.19). The high shear stress values observed for 0.1 and 0.25 M NaOH treated epimysium samples at 55 ºC, compared to low shear stress values observed for other treatments, must have contributed to the interaction temperature*concentration (Table 5.14). At 70 ºC, shear stress was decreased to <5 N/mm^2 but at 55 ºC, a similar low value was observed only with 0.05 M NaOH.

Only the temperature of heating had influenced the shear stress of NH_4OH treated epimysium ($P<0.001$) (Table 5.15). Shear stress of NH_4OH treated epimysium after heating at 55 ºC (Table 5.16) was similar to that of raw epimysium (Table 5.1). As temperature was increased to 70 ºC, a several fold reduction in shear stress was observed (Table 5.16). Interestingly, NH_4OH concentration and the pre-equilibration treatment had no effect on shear stress. Therefore, it was clear that the shear stress reduction observed after combined NH_4OH and thermal treatments was resulting from the latter. Shear stress values of NaOH and NH_4OH treated epimysium were similar to those heated with water at 55 and 70 ºC (Table 5.17). As such, neither heating in NaOH nor in NH_4OH was superior to heating in water under the conditions of the present experiment.

5.5 Supplement

This supplement was prepared to present complementary data on the effect of heating epimysium with acid and alkali (selected concentrations) on thermal denaturation temperature of epimysial proteins, amide bands of proteins and ultra structure in order to verify and clarify observations from the main study.

5.5.1 Differential Scanning Calorimetry (DSC)

Thermal denaturation temperature of epimysium samples equilibrated in de-ionized water over night was considered as the control to compare the changes introduced by treatments. Epimysial proteins heated in (excess) water had shown a peak (mean) thermal denaturation temperature at 63±2 ºC (Table 5.21 and Figure 5.5). Epimysium samples, which were equilibrated overnight in 0.25 M HCl and 0.25 M CH_3COOH did not produce denaturation peaks during heating, indicating the proteins were completely denatured by acids prior to heating (Figure 5.5). Epimysium samples equilibrated overnight in 0.025 M NaOH and 0.25 M NH_4OH had produced thermal denaturation peaks at 64±1.8 and 66±0.5 ºC, respectively (Table 5.22; Figure 5.5).

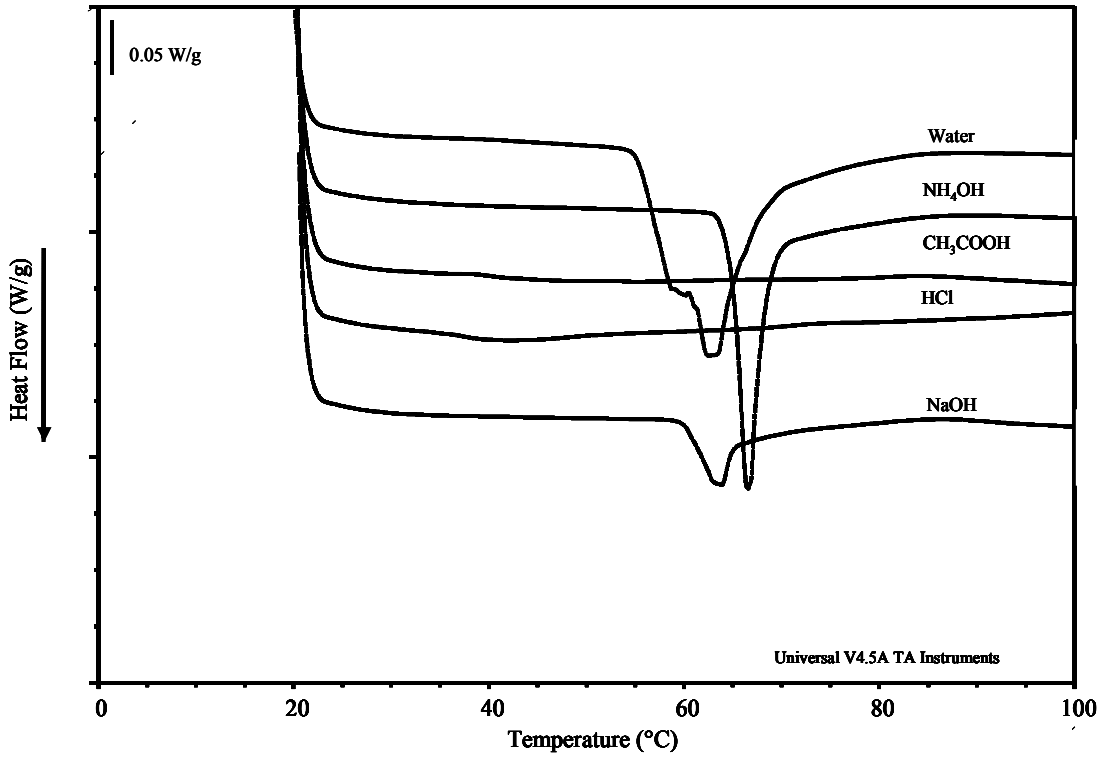


Figure 5.5 DSC thermograms for epimysial proteins pre-equilibrated in HCl, CH_3COOH, NaOH, NH_4OH and water. Ground epimysium samples (5-7 mg) were soaked over night in 0.25 M HCl, CH_3COOH, NH_4OH and 0.025 M NaOH. Heating was carried out at 5 ºC/min starting from 20ºC to 140 ºC but these thermograms were drawn only up to 100 ºC. The absence of peaks after HCl and CH_3COOH treatments indicates that epimysial proteins were denatured prior to heating. Thermal denaturation temperature of protein was not altered after equilibration in water and 0.025 M NaOH. NH_4OH treatment had significantly raised the thermal denaturation temperature of epimysial proteins.

Table 5.22 Comparison of thermal denaturation temperatures of epimysial proteins after NaOH, NH_4OH and water treatments

Treatment	Denaturation temperature peak (ºC)	Onset temperature (ºC)
Water	63.02±2.02	53.25±2.45
0.025 M NaOH	64.02±1.92	55.91±2.58
0.25 M NH_4OH	66.15±0.45	55.97±2.00
P	0.017	0.031
LSD	2.00	2.18

N=6 with 3 sub samples for each replicate. Pre-determined significance level of the experiment was 0.05.
No denaturation peaks were observed after 0.25 M HCl and CH_3COOH treatments.

Thermal denaturation temperatures of epimysial proteins treated in 0.025 M NaOH and water were similar (Table 5.22). Heating epimysium with NH_4OH had clearly increased the thermal denaturation temperature of proteins to higher values than those observed for water and NaOH treated epimysium. This indicated that NH_4OH had increased the thermal stability of the intermolecular bonds of epimysium. The onset temperature for protein denaturation after 0.25 M NH_4OH treated epimysium was significantly higher than that of water treated epimysium but no difference was observed between two alkalis.

5.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

The epimysial matrix consisted of variety of biomolecules, for example, collagen, proteoglycans, elastin, lipids and other minor proteins. However, this discussion was confined to the changes in proteins, specifically to amide bands II and I.

Epimysium heated to 70 ºC for 15 min in water had produced spectra characteristic to amide II (peak at 1550 cm^{-1}) and amide I (1675-1658 cm^{-1}) bands of collagen. These spectra did not show the expected thermal denaturation of collagen (Figure 5.6). The sample treated with 0.25 M HCl and heated to 70 ºC had produced typical amide I bands (1675-1655 cm^{-1}) and also bands with lower wave numbers (Figure 5.7). The latter might be resulting from collagen

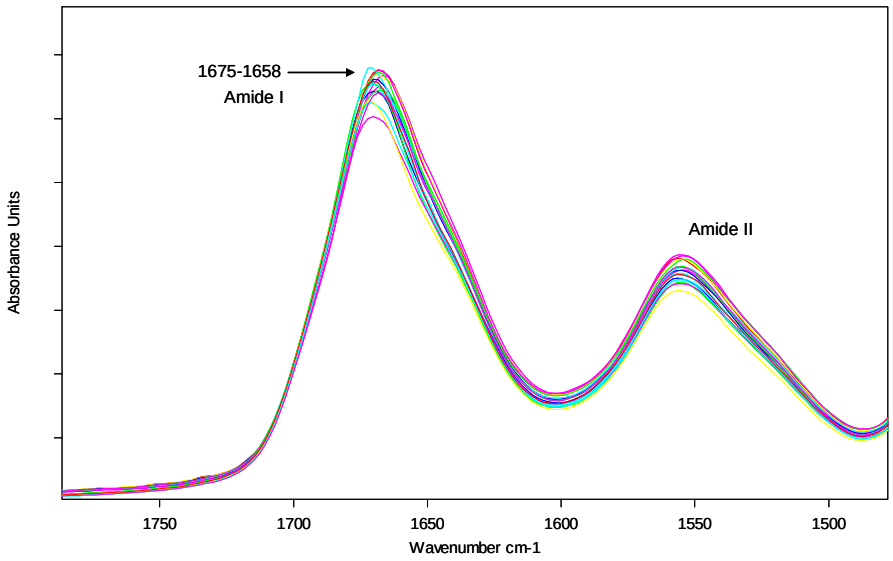


Figure 5.6 IR spectra of epimysium after heating to 70 ºC with de-ionized water. Amide bands I (1675-1658 cm^{-1}) and II (1550 cm^{-1}) typical to proteins are observed despite the exposure to high temperature.

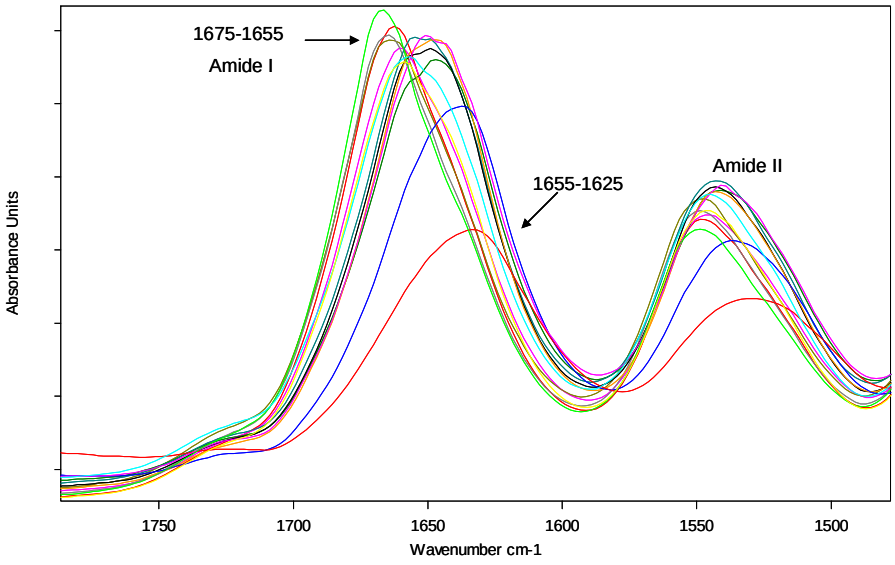


Figure 5.7 IR spectra of bovine epimysium after heating to 70 ºC with 0.25 M HCl. Amide I (1675-1655 cm^{-1}) and amide II bands (1550 cm^{-1}), characteristic to native proteins, are observed together with other bands shifted to low wave numbers.

denaturation. Epimysium, treated with 0.5 M NH_4OH had produced a typical amide I band (1655-1675 cm^{-1}) with a sharp shoulder (1640-1620 cm^{-1}). This shoulder might be representing the irreversibly denatured collagen (Figure 5.8). Spectral splitting was observed for epimysium heated to 120 ºC for 20 min at 20-psi pressure; the two peaks produced had similar absorbance intensities. The amide I band was shifted to higher wave numbers (1682-1664 cm^{-1}) and the second peak (1645-1620 cm^{-1}) might represent the irreversibly denatured collagen (Figure 5.9). Amide II band (peak at 1550 cm^{-1}) was not affected by the aforementioned treatments, except for HCl where the spectra were slightly moved towards the low wave numbers. Unfortunately, spectra of raw epimysium could not be produced due to problems associated with sample sectioning.

5.5.3 Transmission Electron Microscopy (TEM)

Transmission electron micrographs were provided to show the epimysial changes subsequent to heating in water, acid and alkali, despite their poor visual quality. The other reason for their presence in the thesis is unavailability of similar micrographs from previous studies after acid and alkali treatments. These raw epimysium samples were stored at -20 ºC before preparing them for microscopic imaging and that might have contributed to the observed signs of protein degradation (white areas) observed on the images of raw epimysium (Figure 5.10a and Figure 5.10b). The characteristic-banding pattern of collagen fibres (alternate dark and light regions) was seen on cow epimysial collagen fibres before heating (Figure 5.10b). As per micrographs, some inter fibre spacing was observed in cross-sections despite acid, alkali and heat treatments (Figure 5.10c and Figure 5.10f). These collagen fibres might be still carrying some of the original characteristics as observed in IR spectra (Figures 5.6 to 5.10). These observations were compatible with the amide bands I and II of epimysium samples (FTIR data) heated in water (Figure 5.6) and 0.25 M HCl (Figure 5.7), which indicated the presence of some

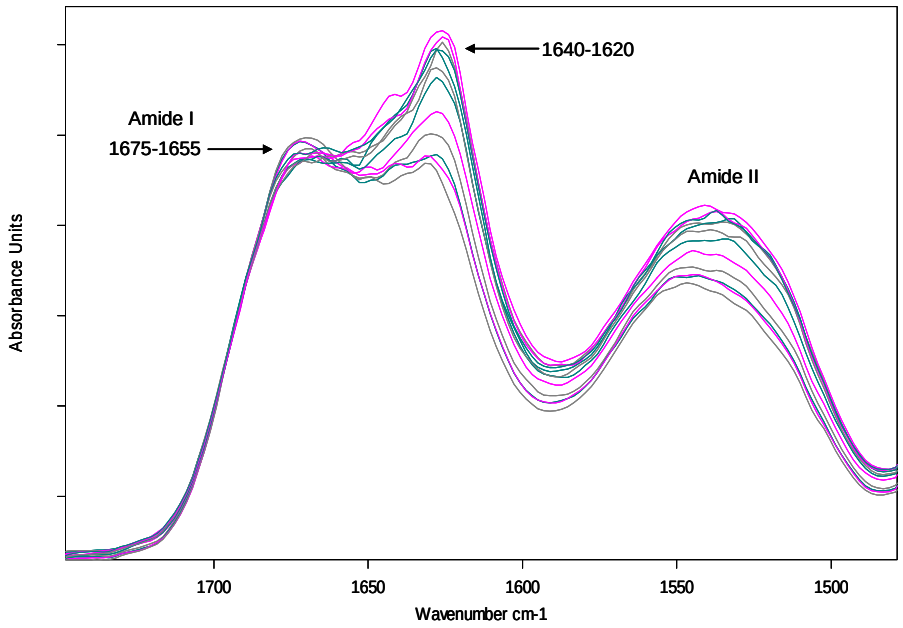


Figure 5.8 IR spectra of bovine epimysium after heating to 70 ºC with 0.25 M NH_4OH. On the shoulder of the characteristic amide I band (1675-1655 cm^{-1}) a new peak emerged (1640-1620 cm^{-1}).

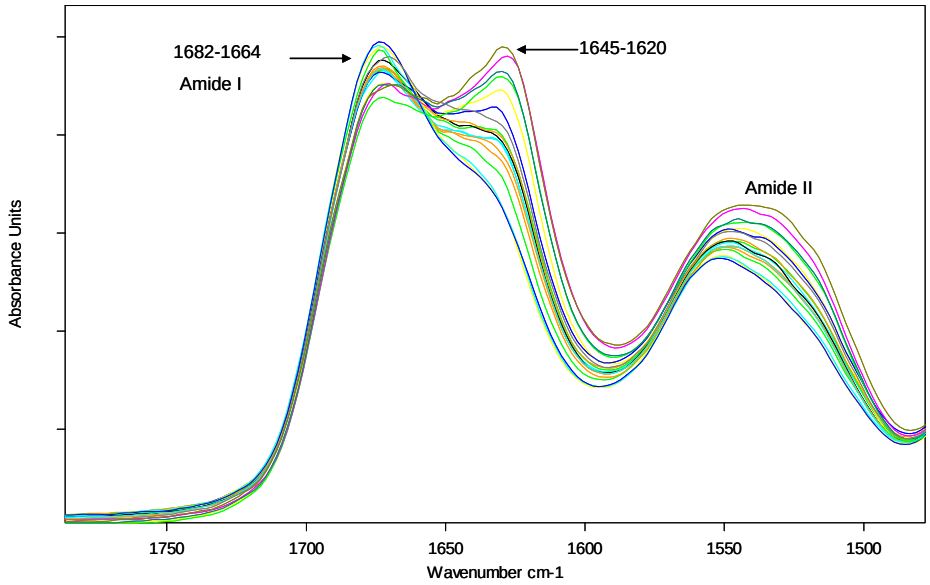


Figure 5.9 IR spectra of bovine epimysium after heating to 120 ºC with de-ionized water at 20-psi pressure. Amide I band was shifted to high wave numbers (1682-1664 cm^{-1}) with a peak splitting to produce a new peak (1645-1620 cm^{-1}).

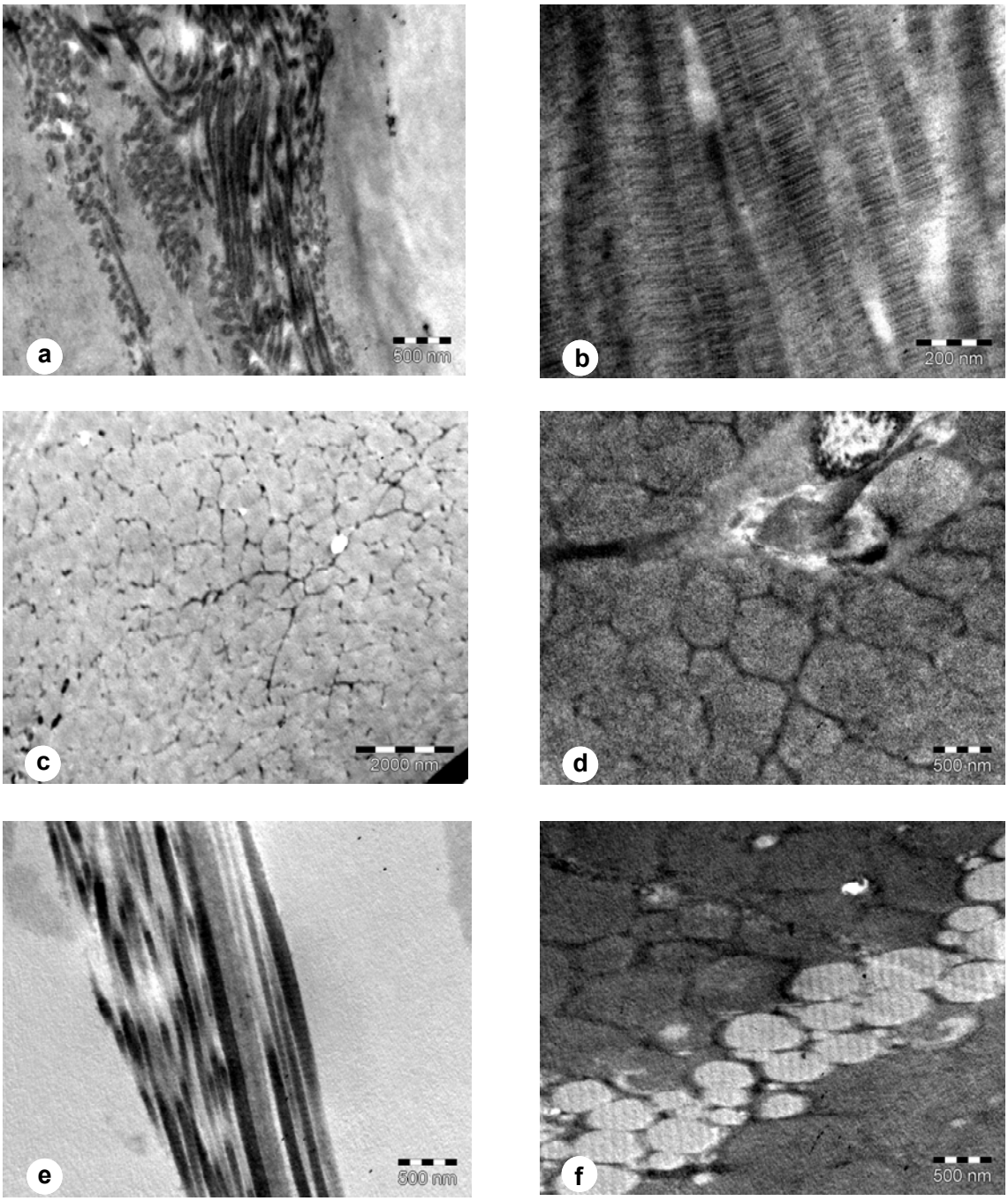


Figure 5.10 Transmission electron micrographs of bovine epimysium before and after heating in acid and alkali. (a) A longitudinal section showing banding pattern on raw epimysial collagen, (b) A longitudinal section of raw epimysial collagen fibre bundles; despite dense packing inter fibre spacing is seen, (c) A cross section of epimysium after equilibrating in 0.25 M HCl for 180 min and subsequent heating to 70 ºC; inter fibre spacing is visible after a severe treatment, (d) A cross-section of collagen fibres after heating in water at 70 ºC, (e) A longitudinal section of epimysium after equilibrating in 0.5 M NH_4OH for 180 min and subsequent heating to 70 ºC; some of the fibres still maintain the original shape, (f) A cross section of 0.5 M NH_4OH treated epimysial collagen.

not denatured proteins. Also, epimysial IR spectra for 0.25 M NH_4OH treated samples had produced the typical amide I bands and had confirmed TEM observations (Figure 5.8). Therefore, it was clear that protein denaturation was not uniformly distributed across epimysium and/or at least some of the epimysial proteins were resistant to the chemical and thermal changes in the microenvironment.

5.6 Discussion

5.6.1 Acid treatments

In one of the early reports on protein hydrolysis by acid and alkali, Mirsky and Pauling (1936) suggested that acids provided protons to electronegative atoms of proteins, which otherwise shared protons among them, and alkali removed protons that were necessary for hydrogen bonding. Out of the two acids used in this experiment, 0.1, 0.25 and 0.5 M HCl dissociated completely to produce 0.1, 0.25 and 0.5 H^+ ion moles/L, respectively.

CH_3COOH dissociated weakly (Ka=1.74 x 10^{-5} at 25 ºC) to produce 1.14 x 10^{-3}, 2.08 x 10^{-3} and 2.95 x 10^{-3} H^+ ion moles/L at 0.1, 0.25 and 0.5 M concentrations, respectively. Therefore, amounts of H^+ ions produced by CH_3COOH were considerably lower than amounts produced by HCl at corresponding concentrations. The Ka, dissociation constant of an acid, was reported to be temperature dependant (Lezina et al., 1981; Sue et al., 2003). The effect of temperature *(T)* on Ka of CH_3COOH was explained by $[d(\ln Ka)/dT=\delta H^o/RT^2]$, in which δH^o was the enthalpy of the system at a given temperature and *R* was the gas constant (Calder and Barton, 1971). As temperature was increased from 5 to 55 ºC, δH^o was reduced from +657 to –901 cal/mol. Therefore, *ln Ka* was reduced. As *ln Ka* was decreased with increasing temperature, Ka was increased to dissociate more H^+ ions (Calder and Barton, 1971). As such, H^+ ion concentration and thus the efficacy of CH_3COOH at 55 and 70 ºC were expected to be higher than values reported at 25 ºC. Very low concentrations of H^+ ions (1 x 10^{-7}) produced by water would exert

little or no effect on proteins in comparison to greater amounts of H^+ ions provided by the two acids under study.

The amounts of protein released by epimysium after acid treatments were followed. As already seen in SDS gels (Figure 5.1 and Figure 5.2), HCl and CH_3COOH hydrolysis of epimysial collagen at 55 and 70 ºC, had produced either single/double strands or short chain peptides. Protein released by HCl treated epimysium was subjected to the combined influence of (interaction) concentration of acid and the temperature of heating (Table 5.2); significantly high amounts of proteins were released as the temperature was increased to 70 ºC with 0.5 M HCl (Table 5.9). The maximum amount of proteins liberated after HCl treatments at 55 ºC was <2% (on weight basis) but this was increased to 10±4.8% (on weight basis) when temperature was raised to 70 ºC together with 0.5 M HCl concentration (Table 5.9). In addition, pre-equilibration of epimysium in HCl (90 or 180 min) prior to heating also had helped to liberate more proteins. Long pre-equilibration times and high temperature had increased the protein liberation from CH_3COOH treated epimysium (Table 5.6) though the maximum amounts released were <2% (Table 5.6). Thus, the effect of CH_3COOH on epimysial protein release at 70 ºC was similar to the effect of HCl at 55 ºC. When these proteins released were considered as a percentage of total protein in epimysium, about 9.6±7.2% and 23±9.7 9% of proteins were liberated with 0.25 and 0.5 M HCl at 70 ºC. All other combinations of HCl concentration and temperature could release <5% of proteins.

Mineral acids such as HCl dissociate peptide bonds in hydrophilic areas of proteins but strong organic acids could act on peptide bonds in hydrophobic areas (Tsugita and Schefler, 1982). At elevated temperatures, the speed of protein hydrolysis was increased (Westall et al., 1972). Therefore, the higher release of epimysial proteins by HCl, in the present experiment, might be resulting from the hydrolysis of peptide bonds in hydrophilic areas of epimysial proteins, mainly

collagen. Collagen was reported to contain an equal proportion of hydrophobic and hydrophilic amino acids, according to the classification that considered all non-polar and aromatic amino acids as hydrophobic and all polar amino acids (both charged and uncharged) as hydrophilic (Ziyatdinov et al., 2006). As temperature was increased from 55 to 70 ºC, energy input also was increased and it was likely that endothermic bonds (mostly hydrogen bonds) in collagen were dissociated. As a result, H^+ ions, supplied by the completely dissociated HCl, must have had an increased opportunity to act on peptide bonds. Even though, collagen contains equal amounts of hydrophobic and hydrophilic amino acids (Ziyatdinov et al., 2006), H^+ ions from the weakly dissociated CH_3COOH had not had the same impact as HCl had on the hydrolysis of peptide bonds. This might be the reason for low release of protein after CH_3COOH treatment at 70 ºC. At 55 ºC, 0.1 and 0.25 M HCl was not superior to CH_3COOH in their ability to release epimysial proteins and were even similar to the effect of water (Table 5.5). Therefore, both HCl and CH_3COOH had little or no effect at 55 ºC. It was thought that heating for 1 h at 55 ºC was hardly sufficient to provide the activation energy required to begin H^+ mediated bond scission and/or to disturb the epimysium matrix to facilitate movement of H^+ into it. At 70 ºC, 0.1 M HCl was superior to 0.5 M CH_3COOH and these treatments had liberated more proteins from epimysium than water (Table 5.5).

In this study, release of OPA reactive amino groups was also measured in addition to proteins. OPA reacts with primary amines (but not with secondary amines) in a basic media to produce a fluorescent complex that absorbs strongly at 340 nm (Benson and Hare, 1975; Janolino and Swaisgood, 1992). Further, proteins released after heating with acid and alkali was analysed using the BCA protein assay. In BCA assay, about 4-6 peptide bonds from tri or polypeptides react with a molecule of Cu^{2+} and reduce to Cu^+. Importantly, dipeptides and amino acids do not react with Cu^{2+} (Smith et al., 1985). (According to this author, a molecule of Cu^+, reacted with 2 molecules of BCA to form a purple-coloured product where the colour intensity was

proportional to the number of peptide bonds reacted). This complex strongly absorbed at 572 nm. Therefore, an overlap existed between the two methods; BCA measured all peptides larger than tripeptides and OPA measured $-NH_2$ groups from peptides of any length including amino acids and non-protein amines. As observed in this study, heating epimysium with HCl at 55 ºC had produced similar amounts of proteins and OPA reactive groups. However, after heating at 70 ºC, the amount of protein released was higher than the amount of OPA reactive groups released (Table 5.9). This indicated that the action of HCl at 70 ºC was mostly confined to the production of polypeptides but not to the production of short chain peptides and amino acids. This was also confirmed by the smears observed in SDS gels (Figure 5.1) due to short chain peptides. Similar results were also observed for NaOH derived proteins and OPA reactive groups after 55 and 70 ºC heat treatments (Table 5.19).

Epimysial weight gain (liquid uptake) after HCl treatment was influenced by the combined effect (interaction) of acid concentration and pre-equilibration time and also the temperature of heating (Table 5.2). In contrast, the ability of epimysial proteins to retain liquid after CH_3COOH treatment was influenced by the increasing concentration of CH_3COOH and longer pre-equilibration times in acid but not by the increasing temperature (Table 5.6). Thus, the temperature of heating had acted differently on epimysial weight gain in the presence of strong and weak acids. Epimysial samples had gained the maximum weight after 180 min pre-equilibration in 0.1 M HCl and subsequent heating to 70 ºC (180±28%) (Table 5.4). As the HCl concentration was increased (to 0.5 M) at 70 ºC, weight gain was decreased to ~22-25% (Table 5.4). Epimysial weight gain after 180 min pre-equilibration in CH_3COOH was (134±39%) similar to values observed for HCl (Table 5.7). However, these observations did not agree with earlier reports by Bowes and Kenten (1950). Accordingly, aqueous phase uptake by collagen in acid solutions at 20 ºC was a result of osmotic pressure differences and collagen had gained 6 and 8 times of its original weight at pH 2 and pH 13, respectively. Collagen bound to the

epimysial matrix might not act the same way as isolated collagen did in Bowes and Kenten's (1950) experiment. Therefore, epimysial weight gain data were reviewed in the light of epimysial protein release.

As already observed, the maximum amount of protein released after CH_3COOH treatments was <2% (on raw weight basis) (Table 5.7) and the corresponding value for HCl was about 10±4.8% (on raw weight basis) (Table 5.9). As such, release of high amounts of epimysial proteins after HCl treatments might have contributed to the observed reduction in weight gain. Further, the observed negative effect of increasing concentration of HCl, longer pre-equilibration times and increasing temperature on weight gain/absorption of aqueous phase might be a result of H^+ ions from HCl, being strong electron acceptors, interacting with proteins at all exposed sites to prevent them binding water through hydrogen bonding. Weakly dissociating CH_3COOH had produced a lower concentration of H^+ (thus, weak electron acceptors) and was not equally competitive as H^+ ions from HCl to prevent binding of water by proteins.

Thickness increase demonstrated a uniaxial change in epimysium and which explained part of the process leading to collagen swelling (a volume change). It was thought that charged ions of acid and alkalis (+ and – ions) entered into the epimysium and had neutralized the opposite charges on proteins. Thus, accumulation of one charge over the other on protein surfaces leads to repulsion between charged surfaces. Decreased attraction was thought to be the cause of epimysium thickness increase. The degree of increase in epimysial thickness gradually became smaller as a result of the combined effect (interaction) of increasing concentration of HCl, longer pre-equilibration times and increasing temperature (Table 5.2). IR spectra of epimysial samples pre-equilibrated in 0.25 M HCl and subsequently heated to 70 ºC for 180 min had shown the presence of typical amide I spectra together with displaced spectra (1675-1625 cm^{-1}) (Figure 5.7). These displaced spectra may be an indication of the localized denaturation of

collagen by HCl but with some undenatured areas. Perhaps, localized denaturation may be a result of inadequate pre-equilibration time (treatment applied was 180 min) to penetrate acid into the inner layers of epimysial matrix. It was also thought that, this unaltered IR spectra were resulting from re-naturation of collagen exposed to heating as explained by Hörmann and Schlebusch (1971). Accordingly, only the collagen irreversibly denatured could produce an altered IR spectrum. The highest thickness change (266±72%) was attained at 180 min pre-equilibration in 0.1 M HCl and subsequent heating to 70 ºC. The lowest thickness value (27±6%) was attained at 180 min pre-equilibration in 0.5 M HCl and subsequent heating to 70 ºC (Table 5.4). Epimysial thickness change had followed the same trend as weight gain after HCl treatments. Therefore, these two parameters might be influenced by the same factors, perhaps exposure of proteins to acid and subsequent loss of protein. The thickness change of CH_3COOH treated epimysium was increased with the increasing concentration of CH_3COOH, pre-equilibration time and temperature (Table 5.6). A similar increasing trend was observed for epimysial weight gain with the increasing concentration of CH_3COOH and pre-equilibration time. However, epimysial weight gain after CH_3COOH treatment was not different at 55 and 70 ºC heat treatments. The maximum thickness change attained by CH_3COOH treated epimysium (mean at any combination of treatments) was <165% of the original thickness (Table 5.7). Therefore, it was clear that 0.1 M HCl had a better ability to increase epimysial thickness change than any concentration of CH_3COOH.

Thermograms of (ground) epimysium following overnight exposure to 0.25 M HCl and CH_3COOH did not produce denturation peaks during heating from 20 to 140 ºC. While admitting that conditions of the DSC study were not identical to the rest of the experiment, it was concluded that as the epimysial matrix effect on collagen was minimized (by fine grinding to increase the acid contact surface area) long pre-equilibration treatments (overnight) in HCl and CH_3COOH could completely denture collagen without a heat treatment. For certain, long

pre-equilibration treatments in HCl had provided sufficient time for the (freely available) H^+ to penetrate into the epimysial matrix to act on collagen prior to heating. This denaturation might have been induced by any of the following reasons, pH effect on collagen, disintegration of peptide bonds by H^+ or due to a combination of both. However, previous experiments, which used soluble collagen, had shown that acetic acid could decrease the thermal denaturation temperature to 43 ºC (Friess and Lee, 1996).

Epimysial shear stress was significantly influenced by the combined effect of heating temperatures and the pre-equilibration treatments in two acids (Table 5.2 and Table 5.6). As the pre-equilibration time in HCl and CH_3COOH was increased (from 0 to 90 min) and samples were subsequently heated at 70 ºC, shear stress was reduced to values lower than those observed at 55 ºC for similar treatments. Increasing pre-equilibration time from 90 to 180 min in CH_3COOH had no clear effect on shear stress reduction at 55 and 70 ºC. A similar change in pre-equilibration time with HCl treatment and subsequent heating to 70 ºC had produced shear stress values lower than those observed for CH_3COOH at the same temperature (Table 5.9 and Table 5.12). At 55 ºC, shear stress data from analogous treatments of HCl and CH_3COOH were similar. In other words, HCl treatment was not superior to CH_3COOH treatment at 55 ºC. It was also noted that at 55 ºC and at 0 min pre-equilibration treatment, neither HCl nor CH_3COOH was better than water in decreasing shear stress. Also, increasing concentration of HCl had decreased the epimysial shear stress values significantly.

Comparing Warner-Bratzler shear force data (in these experiments, force data were not corrected for the area of the meat surface) and consumer perceived toughness data of meat (taste panel data), it was concluded that consumers could detect a toughness difference of about 9.81 N (1 kg) and 4.9 N (0.5 kg) in restaurant and home settings, respectively (Miller et al., 1995). Huffman et al. (1996) also supported that a difference of 1 kg was noticeable to consumers. A

consumer rating of 'very tender' was reported for meat samples having shear force values of <32.96 N for a diameter of 1.27 cm; that is similar to a shear stress value of 0.26 N/mm^2 (Destefanis, 2008). While considering that consumers would not perceive sensory properties of meat and epimysium in the same way, it was clear that none of the acid treatments could bring down the shear stress values to standards set by Destefanis (2008).

Whiting and Strange (1990) had observed that acid treatments (0.5 M HCl and CH_3COOH at 22 ºC) alone could not reduce the shear strength of raw bovine epimysium. Based on the current experimental data, it could be concluded that HCl and CH_3COOH concentrations to ≤ 0.5 M and together with heating at 70 ºC for 15 min could not reduce shear stress values of cow epimysium to low values attained by meat under normal cooking. Despite that, the original aim of this experiment was to reduce shear stress values of cow epimysium to <2 N/mm^2, the lowest values reported for heifer epimysium after aqueous heating in a previous experiment. This target was achieved only after 180 min pre-equilibration in HCl and subsequent heating to 70 ºC. Therefore, dilute CH_3COOH was useful to reduce shear stress of connective tissue when combined with pre-equilibration treatments but could not decrease shear stress to the target set in the present experiment. According to previous reports, CH_3COOH was a useful muscle protein tenderizer at ordinary cooking temperatures, for example, 71 ºC for 2 min (Burke and Monahan, 2003). On the other hand, as the HCl concentration was increased to 0.25 M and subsequently heated to 70 ºC about 9.6±7% of epimysial proteins were liberated while about 90 % proteins were still in the matrix (Table 5.10). Despite the high percentage of proteins retained, shear stress was reduced to <5 N/mm^2 (data not shown). This showed that protein retained in epimysium had little or no effect on shear stress values. However, IR spectra did not show signs of complete denaturation of epimysial proteins after the 180 min pre-equilibration treatment in 0.25 M HCl and subsequent heating to 70 ºC. Therefore, it was hypothesised that shear stress decrease under the conditions of this experiment was resulting from H^+/Cl^- mediated

neutralization of charges on proteins and resulting lack of attraction between and among protein layers facilitating entry of water. This hypothesis was supported by the higher weight gain data observed after 0.1 M (~179%) and 0.25 M HCl (~89%) treatments with a 180 min pre-equilibration treatment.

Based on the overall observations regarding the functions of two acids, the hypothesis, 'a strong proton donor has a better capacity to weaken the epimysium structure than a weak proton donor through the hydrolysis of bonds of the collagen', needed to be amended. The ability of HCl to modify the epimysium matrix more than CH_3COOH did, was a function of the strong proton donation ability of HCl but this was undoubtedly supported by the combined effects of concentration of HCl, pre-equilibration time and the final temperature of heating. Precisely, weight gain and thickness change were supported by the combined effect of all three treatments (listed above) but shear stress reduction was facilitated by the combined effect of temperature and the pre-equilibration time in HCl. The combined effect of HCl concentration and the temperature of heating supported the amount of protein liberated from epimysium. In a practical point of view CH_3COOH also could decrease shear stress/raw thickness of epimysium when combined with long pre-equilibration times and subsequent heating at 70 ºC but was not as effective as HCl.

5.6.2 Alkali treatments

NaOH is a strong base and was expected to produce 0.01, 0.025 and 0.05 M of OH^- ions through the complete dissociation of the alkali. NH_4OH is a weak base (Kb= 1.79 x10^{-5} at 25 ºC) and 0.1, 0.25 and 0.5 M concentrations of NH_4OH were expected to produce 1.33 x 10^{-3}, 2.11 x 10^{-3} and 2.99 x10^{-3} moles/L OH^- ions at room temperature. Concentrations of NaOH used were 10 times lower than that of NH_4OH but OH^- concentrations of NaOH were higher than that of

NH_4OH. The effect of OH^- ions produced by water (1×10^{-7} at 25 ºC) was insignificant compared to the OH^- ions produced by the two alkalis.

During heating with two alkalis, epimysium had released proteins and the amounts were significantly influenced by the concentration of alkali and the pre-equilibration time (Table 5.13 and 5.15). Thus, the higher the concentration of alkali and the higher the length of the pre-equilibration treatment the more proteins were liberated (Table 5.16 and Table 5.19). The temperature of heating had no effect on protein release after alkali treatments (Table 5.13 and Table 5.15). Subject to the experimental conditions, the amounts of protein released from epimysium after NaOH and NH_4OH treatments were similar and represented <2% of the weight of epimysium and <4.5% of the total protein in epimysium (Table 5.16 and Table 5.19). Except for 0.5 M NH_4OH (without a pre-equilibration treatment) at 70 ºC, none of the alkali treatments (without a pre-equilibration treatment) were superior to heating in water in their ability to release protein (Table 5.17). Similar to acid treatments, alkali also released single and double strands of collagen together with other unidentified proteins. The protein bands on SDS gels (Figure 5.3 and 5.4) also provided evidence for the relative effectiveness of NaOH and NH_4OH treatments after 180 min pre-equilibration in alkali. The absence of single and double strands of collagen after 0.05 M NaOH treatment, when these bands were present after 0.5 M NH_4OH treatment, had indicated the strong protein hydrolysis effect of NaOH treatment.

Free amino groups released after NaOH treatment was measured as OPA reactive groups and discussed in detail under acid treatments. The release of OPA reactive free amino groups was increased as the concentration, pre-equilibration time and temperature of heating were increased (Table 5.13 and Table 5.19). The amount of OPA reactive groups were always less than the amount of protein liberated. As explained under acid treatments, this must be resulting from release of fewer short chain peptides and amino acids than polypeptides.

Due to the limitations of the analytical method, release of OPA reactive groups from epimysium heated in NH_4OH could not be measured. As observed, the reaction among NH_4^+/NH_3, *o*-phthalaldehyde and dithiothreitol had immediately produced a pale yellow colour, which gradually intensified to dark orange while absorbance at 340 nm was continued to drop indicating an ongoing chemical transition in the system. The latter observation was compatible with the explanations given in a comprehensive study on the reaction of OPA with NH_3/NH_4^+ (Kulla and Zuman, 2008). According to them, OPA and NH_4^+/NH_3 reaction followed through three phases; a vary rapid reaction within the first 2 min at room temperature, a slow reaction followed within next 5 to 60 min and lastly a very slow reaction which began after 60 min to generate products that absorbed at wavelengths >350 nm. This sequence of reactions involved many side reactions and made thirteen different products, some of which were stable and others that were unstable (Kulla and Zuman, 2008). According to Sugawara and Oyama (1981) NH_3 from NH_4OH reacted with *o*-phthalaldehyde to form fluorescence at λ_{ex} =413 nm and λ_{em}=476 nm and α-amino acids produce fluorescence at λ_{ex} =339 nm and λ_{em}=468nm in the presence of dithiothreitol. As such, amino acids present in NH_3 rich media could be distinguished from the others at the correct excitation wavelength. However, Sugawara and Oyama's (1981) experiment did not comply with the observations of the present study. Therefore, it was concluded that the *o*-phthalaldehyde method was not suitable to detect free amino groups in the presence of NH_3 or NH_4^+ compounds.

The combined effects of pre-equilibration time and concentration had influenced the epimysial weight gain after NaOH treatment (Table 5.13); 180 min pre-equilibration in 0.05 M NaOH had increased the weight gain to about 74±8% (Table 5.14). In addition, a significant increase in epimysial weight gain was noted with NaOH, when the temperature was increased from 55 ºC to 70 ºC. With the increasing concentration of NH_4OH, pre-equilibration time and temperature of

heating epimysium weight gain was increased (Table 5.15 and Table 5.16). However, the maximum weight gain after heating in NH_4OH was about 50%.

It was reported that water absorption by isolated collagen increased from pH 7 to pH 13.4 with a plateau observed between pH 11.5 and 13 (Bowes and Kenten, 1950). Consequently, weight of isolated collagen was increased from 400 to 500% when pH was increased from 11 to 12 and without a heat treatment (Bowes and Kenten, 1950). The two alkali used in the current experiment resulted in pH values that lay in the plateau region (mentioned above). The collagen dominant epimysium did not gain weight as demonstrated by Bowes and Kenten (1950), however the present experiment was different as it used a tissue (epimysium) and also included a heat treatment (15 min at 70 ºC). The maximum weight gain reported after heating epimysium with 0.05 M NaOH at 70 ºC was 74±8% (Table 5.14). This observed low weight gain might be a result of the matrix effect; collagen bound to the other biomolecules of the matrix might not have the same freedom to imbibe liquid as isolated collagen did.

Thickness of epimysium was another parameter affected by the treatments. Increasing concentration of NaOH had increased the thickness of epimysium and the maximum thickness (more than double of the original thickness) was attained at 70 ºC (Table 5.13 and Table 5.19). The combined effect of temperature and pre-equilibration time had influenced the epimysial thickness increase after NH_4OH treatment (Table 5.15). Heating in NH_4OH at 55 ºC had hardly any effect on epimysial thickness increase irrespective of pre-equilibration treatment (~12-14%) but as the temperature was increased to 70 ºC thickness was increased more than double of the original thickness (Table 5.18). Without pre-equilibration, neither NaOH nor NH_4OH treatment was superior to heating epimysium in water in their ability to increase thickness (Table 5.17). The thickness change for NaOH and NH_4OH treated epimysium were in the range of 34-100% and 12-108%, respectively (Table 5.19 and Table 5.20).

It was considered important to link the observations from TEM micrographs with the thickness data. Epimysium pre-equilibrated in 0.5 M NH_4OH for 180 min and subsequently heated to 70 ºC for 15 min, had contained inter fibre spacing (Figure 5.6 e and f). However, previous investigators had reported that voids among fibres were eliminated due to shrinkage of fibres, increased fibre diameter and fusion of fibres subsequent to heating (Wöhlisch, 1932; Van Hook, 1947; Haly et al., 1971; Kirsch et al., 1998; Ma et al., 2005).

After NaOH treatment, shear stress was influenced by the combined effect of heating temperature and the concentration of alkali (Table 5.13). High shear stress values were observed after 0.01 and 0.025 M NaOH treatments at 55 ºC but then shear stress was decreased (<5 N/mm^2) as the NaOH concentration was increased to 0.05 M at 55 ºC and also as the temperature was increased to 70 ºC (Table 5.14). After heating epimysium in 0.05 M NaOH, the maximum amount of protein liberated from epimysium was <5% of the total protein (Kjeldahl). In other words, ~95% proteins (on protein basis) were retained in the epimysium. It was clear that the quantity of proteins retained had little effect on the epimysial shear stress because it was already decreased to <5 N/mm^2. Shear stress of NH_4OH treated epimysium was not decreased at 55 ºC (Table 5.16), however, as the temperature of heating was increased to 70 ºC, shear stress was significantly decreased (Table 5.15 and Table 5.16). Shear stress was not influenced by the concentration of NH_4OH and pre-equilibration time in it (Table 5.15). Thus, without hesitation it could be said that observed reduction in shear stress values was resulting from the exposure to high temperature. Similar to NaOH, the lowest observed values of shear stress of NH_4OH treated epimysium were in the range of 5-7 N/mm^2. But neither NaOH nor NH_4OH could decrease the shear stress to the target set at the beginning the experiment (2 N/mm^2).

It was considered important to draw attention to shear stress data of NH_4OH treated epimysium at 55 ºC. Shear stress values of NH_4OH treated epimysium (38.9±3.5 N/mm^2) obtained at 55 ºC were similar to that of the raw epimysium (38.4±6.8 N/mm^2) (Table 5.16 and Table 5.1). This indicated that either NH_4OH had no effect at all on epimysium or it had an epimysium stabilizing effect, which was not eliminated after heating at 55 ºC. However, under similar conditions of heating, shear stress values of NaOH treated epimysium were considerably reduced due to the combined effect of increasing concentration of alkali and the pre-equilibration time (Table 5.13 and Table 5.14). As such, it was hypothesised that NH_4OH had an epimysium stabilization effect and heating at 55 ºC did not eliminate that effect. This hypothesis was supported by the DSC data. Thermal denaturation temperature of 0.25 M NH_4OH treated epimysium was 3 ºC higher than (66±0.45 ºC) the epimysium samples stabilized in (excess) water and also 2 ºC higher than samples stabilized in NaOH (Table 5.22). Because, shear stress of NH_4OH treated epimysium was decreased after heating at 70 ºC, it was thought that epimysium stabilization effect of the alkali was eliminated after heating to higher temperatures.

An intriguing question to discuss was, 'why did NH_4OH stabilize the collagen dominant epimysium?' According to one observation, elastin incubated with NH_4Cl for 7-28 days at 37 and 60 ºC had formed pyridine cross-links and it was thought to be due to condensation of allysine with NH_3 (Umeda et al., 2001). Even though allysine (a primary product of collagen cross-linking pathway) was present in collagen, it was unlikely to form cross-links within the short pre-equilibration times employed in this experiment. On the other hand, if this stabilization effect was a result of cross-linking then those cross-links must be thermolabile at 70 ºC. According to Herbine and Dyke (1985) and Nelson et al. (1987), there was no evidence to show that $NH_3.H_2O$ was an ionic complex ($NH^{4+}.OH^-$) or that the proton from H_2O was partly

transferred to NH_3. Ammonia was recognized as a powerful and universal proton acceptor, accepting protons from the weakest donors and having a low tendency to donate to hydrogen bonds (Herbine and Dyke, 1985; Nelson et al., 1987). Further, in an $NH_3.H_2O$ complex, NH_3 formed strong and linear hydrogen bonds with H_2O, where water donated protons. The $NH_3.H_2O$ complex was stronger than a $H_2O.H_2O$ complex because the atomic distances were shorter in $NH_3.H_2O$ complex (Herbine and Dyke, 1985).

The above information together with the observations of the present study provided the ground for a sound argument; NH_3 strongly hydrogen bonded to OH groups of exposed proteins and also to H_2O molecules encapsulating collagen molecules and this strong bond did not dissociate at the energy level provided during heating at 55 ºC heating for 15 min. However, bond dissociation was likely at the energy level provided during heating at 70 ºC for 15 min. The epimysial stabilization effect of NH_4OH was also apparent from the reduced standard deviation for shear stress compared to that of NaOH treatment at 55 ºC. In NH_4OH treated epimysium, shear stress decrease was not influenced by the concentration of the alkali and pre-equilibration treatment but only influenced by the temperature of heating (Table 5.15). These observations did not match the conclusions made in other experiments on meat tenderness (Hamling and Calkins, 2008; Hamling et al., 2008). Accordingly, muscles (bovine round and chuck muscles including the connective tissue rich *biceps femoris*) injected with brine containing NH_4OH and salt and subsequently cooked to 70 ºC had decreased shear force values, toughness values and connective tissue contents (from sensory evaluation) (Hamling and Calkins, 2008; Hamling et al., 2008). Based on the findings of the present study, it was thought that NH_4OH could not be responsible for the shear force reduction in connective tissue but cooking at 70 ºC (and perhaps other brine components) must have decreased it. The original hypothesis set for the present experiment, ‘completely ionized alkali has a better capacity to weaken the epimysium structure

than a weakly dissociated alkali' could not be investigated and verified because the effect of NH_4OH on epimysium did not fit the hypothesis.

The effect of NH_4OH treatment on epimysial collagen at 70 ºC could be elaborated on IR spectra (Figure 5.8). A new peak was emerged (1640-1620 cm^{-1}) on the shoulder of the amide I band (1675-1655 cm^{-1}) as a result of heating epimysium in NH_4OH. Amide I band of β sheets were also noted at 1640-1620 cm^{-1} but could not be clearly distinguished for their arrangement as parallel or anti-parallel sheets (Surewicz et al., 1993). IR spectra of random coils (peptide strands) were noted at 1640 cm^{-1} (Martinez and Millhauser, 1995). Therefore, this new peak was laid in the zone designated for both random coils and β-sheets. Because, the fractional areas under amide I bands for α-helices to β-sheets was thought to be representing percentages of these structures in the protein (Byler and Susi, 1986) it was important to support the spectra with complementary information prior to make judgements. It was rational to speculate that some of the α-helices of collagen might have transformed to β-sheets in the presence of NH_4OH because thermal denaturation temperature of NH_4OH treated epimysium was 3 ºC higher than samples heated in water and 2 ºC higher than samples heated in NaOH. Conversely, already established scientific evidence did not support such a transformation in collagen. The amino acid composition of collagen was dominated by glycine (1/3 of amino acids), proline (1/8) and alanine (1/9) (Eastoe, 1967). Proline and glycine were found to be having the lowest propensity to form β-sheets out of 20 amino acids and alanine also had shown a very low tendency, proline<glycine<alanine (Smith et al., 1994). Further, replacing a standard β-sheet with these three amino acids had reduced the thermal denaturation temperature of the protein considerably. For example, proline brought the thermal denaturation temperature below 10 ºC, glycine brought it to 45.95 ºC and alanine brought it to 57.05 ºC (Smith et al., 1994). As explained already, even though NH_4OH had an epimysium stabilization effect which was not eliminated after heating at 55 ºC, after heating at 70 ºC, shear stress was decreased and stabilization effect was eliminated.

As such, it was concluded that the observed new peak (1640-1620 cm^{-1}) was a result of random coils formed during thermal denaturation of epimysial collagen. Shifting amide I band to lower wave numbers (1625 cm^{-1}) was considered as an indication of some helices converting to random coils (Torikai and Shibata, 1999). Random coil formation was further supported by the presence of a similar peak (1640-1620 cm^{-1}) on the shoulder of amide I band of epimysium heated to 120 ºC for 20 min at 20 psi pressure (Figure 6.6 d). Further, autoclaving was an established method for collagen hydrolysis during elastin recovery (Neuman and Logan, 1950).

A discrepancy observed in IR spectra was that after heating epimysium to 70 ºC in water, a typical amide I band (1675-1658 cm^{-1}) appeared and signs of protein denaturation were not observed. A typical amide I band of collagen was reported to be asymmetric and located at 1650-1665 cm^{-1} and amide II band is located at 1530-1550 cm^{-1} (Kamińska and Sionkowska, 1996). Bands in this region were generally assigned to α-helical structure (Surewicz et al., 1993). Amide I band was thought to be resulting from C=O stretch and amide II band was due to stretching of N-H and C-N bonds (Camacho et al., 2001). Because both C=O and N-H participate in hydrogen bonding in secondary structure, changes there in were expected to reflect in IR spectra. However, the absence of evidence for random coils in IR spectra of collagen heated to 70 ºC with water might be resulting from renaturation of (some of the) amorphous collagen to helices during cooling and freezing as explained by many authors (Garret and Flory, 1956; Veis and Cohen, 1960; Hörmann and Schlebusch, 1971). This indicated that, while collagen in some areas had undergone irreversible thermal transition, collagen in other areas could either maintain its original form after aqueous heating at 70 ºC or reversibly denatured so that it could not be observed after cooling. Also, it is evident that random coils formed after acid and alkai treatments and autoclaving were not renatured during freezing of epimysium samples.

Comparison of results from the two acids and NaOH treatments was difficult due to differences in concentrations used. The overall trend of protein release was such that, none of the three treatments (HCl, CH_3COOH and NH_4OH) was superior to heating epimysium in water at 55 ºC and the maximum amount of protein released was <2% (of the raw epimysial weight). However, the ability of HCl to release protein at 70 ºC was superior to CH_3COOH and NH_4OH. Epimysial shear stress after HCl and CH_3COOH treatments at 55 ºC were similar (mean 6-8 N/mm^2) but NH_4OH had an epimysium toughening effect, which was not eliminated after heating at 55 ºC. After heating at 70 ºC, HCl had decreased shear stress more than CH_3COOH. For example, shear stress values after 180 min pre-equilibration in CH_3COOH and subsequent heating to 70 ºC were similar to heating in HCl (at 70 ºC) with no pre-equilibration treatment (~6 N/mm^2). NH_4OH concentration or pre-equilibration in it had no effect on shear stress decrease.

5.7 Summary and conclusions

In general, each different acid and alkali used in this study had significantly different effects on the properties of cow epimysium. The combined effect of temperature and concentration of HCl determined the amount of epimysial proteins liberated after HCl treatment. Also, long pre-equilibration treatments in HCl had favoured liberation of more epimysial proteins. The temperature of heating and the length of pre-equilibration treatment had determined the protein release after CH_3COOH treatment. After heating in CH_3COOH, the highest amount of protein released was <3.4±1.4 % (protein basis). However, 0.5 M HCl at 70 ºC could release about 23±8% of the epimysial proteins. Concentration of alkali and the pre-equilibration time had governed the protein release after treatments with the two alkalis. The maximum amount of protein released after any of the combinations of alkali treatments was about 4% (on protein basis). Interestingly, temperatures used in this study (55 and 70 ºC) did not influence protein release after alkali treatments. It was noted that, OPA reactive amino groups released from

epimysium after acid and alkali (NaOH) treatments were always less than the protein measured as BCA reactive groups. Based on OPA and BCA assay data, it was thought that after acid and alkali treatments more polypeptides were liberated than dipeptides and amino acids. OPA method was considered not appropriate to measure amino groups in the presence of NH_3 or NH_4^+. IR spectra had shown that after HCl and NH_4OH treatments (some of the) epimysial proteins still maintained an amide band I typical of undenatured proteins. This might be resulting from renaturation of collagen or simply the resistance of those proteins to denature under the conditions provided. Epimysium weight gain (%) and thickness (%) were increased subsequent to acid and alkali treatments due to the individual effects of or combined effects of concentration, pre-equilibration time and temperature; however, HCl was an exception. Weight gain and thickness increase were decreased with the increasing concentration of HCl, pre-equilibration times and temperature.

Shear stress values of HCl and CH_3COOH treated epimysium were decreased due to the combined effect of temperature of heating and the pre-equilibration time in acid. Shear stress was decreased to <2 N/mm^2 (the target set) after a 180 min pre-equilibration treatment in HCl and subsequent heating to 70 ºC. At this point, epimysium had contained about 77% of its proteins (still attached to the matrix), thus it was clear that proteins retained in the epimysium had no effect on shear stress decrease after HCl treatment. After a similar treatment in CH_3COOH, shear stress was decreased to ~5±4 N/mm^2. At 55 ºC, two acids had a similar effect on shear stress data. Shear stress was observed to decrease also with the increasing concentration of HCl. IR spectra of 0.25 M HCl treated epimysium showing typical amide bands while shear stress was considerably decreased had indicated that shear stress reduction was resulting from reasons other than changes in protein structure, most likely H^+/Cl^- mediated neutralization of charges on proteins and subsequent entry of water. At 70 ºC, NaOH had an increased ability to bring down shear stress more than NH_4OH; for example, 0.05 M NaOH at

70 ºC had decreased the shear stress to 3±1.5 N/mm^2 and the lowest values reported after NH_4OH treatment at 70 ºC was 5±2.5 N/mm^2. After heating at 55 ºC, shear stress values of NH_4OH treated epimysium were similar to that of raw epimysium (38±7 N/mm^2). Thermal denaturation temperature of epimysial proteins (collagen) was increased to 66 ºC after NH_4OH treatment, which was significantly higher than the values observed after heating epimysium in water (63 ºC) and NaOH (64 ºC). Clearly, NH_4OH had a collagen stabilization effect but which was eliminated after heating to 70 ºC.

Overall, it was concluded that shear stress of epimysium could be substantially reduced with following treatment combinations; long pre-equilibration time in HCl and CH_3COOH and subsequent heating to 70 ºC, and higher concentration of NaOH and heating to 70 ºC (compared to the raw shear stress values). Higher concentration of NH_4OH and long pre-equilibration times in it had no effect in epimysial shear stress reduction. Other physicochemical properties measured, such as weight gain, thickness change or protein release were not useful indicators to explain the phenomenon of shear stress reduction.

6. GENERAL DISCUSSION

The overall objective of the research leading to this thesis was to understand the contribution of connective tissue to beef toughness and to find means to decrease the effect of connective tissue on toughness. To this effect, intramuscular connective tissue (IMCT) in selected muscles was quantified during the first phase of the research. As IMCT content varied with the physiological needs of muscles and shear force was not related to the quantity of IMCT in all muscles, the physicochemical properties of connective tissue were examined to explore the underlying causes of connective tissue driven beef toughness. Due to its abundance, epimysium was selected as the tissue for investigation. This phase of the study was driven by the hypothesis that 'the phase transition of collagen to attain an amorphous state is the reason for the decreased shear stress of epimysium after heating'. Having understood that aqueous heating could not decrease the shear stress values of cow epimysium to that of heifer epimysium reached under similar conditions of heating, the effect of protein bond scission on shear stress was investigated. Therefore, the last phase of the study was aimed at collagen bond scission in order to decrease shear stress and to reach the overall goal. Both strong and weak acids and alkalis were employed during this phase with the hypothesis that 'a strong proton donor (and also strong alkali) has an increased capacity to weaken the epimysium structure than a weak proton donor (and also weakly dissociating alkali) through the hydrolysis of bonds of collagen. Indicators of protein denaturation and weakened epimysial structure were monitored as appropriate to each phase of the study to relate with the shear force (or shear stress for epimysium), which is a mechanical measure of toughness. The significance of these experiments was the use of epimysial matrix bound collagen to assess the effects of aqueous heating as well as effects of heating in acids and alkalis. Similar studies in the past had used isolated collagen (Bowes and Kenten, 1950; Flory

and Garret, 1958) or other tissues such as rat tail tendon to measure birefringence or enthalpies of phase transition (Miles et al., 1995; Maitland and Walsh, 1997), joint capsular tissue for histological properties after heating (Hayashi et al., 1997), bones to measure collagen denaturation after heating (Wang et al., 2001) and so on.

The intramuscular connective tissues of (selected) bovine muscles were physically separated and quantified for the first time. For this quantification of IMCT, *gluteus medius* (GM), *biceps femoris* (BF), *semimembranosus* (SM) and *longissimus* (L) muscles from both cows and heifers were used. Organized in descending order, IMCT contents of muscles were as follows. GM had the highest amount of IMCT. BF muscles had IMCT contents higher than that of SM muscles. On the other hand, SM and L muscles had similar amounts of IMCT. Among the muscles GM and BF had the highest and second highest contents of IMCT on dry matter basis (9.4 and 7.8%, respectively) and the lowest contents of IMCT were extracted from SM and L (5.1 and 4.2% on dry matter basis, respectively). IMCT content had significantly varied between muscles, most likely to serve the functional needs of muscles and also between cows and heifers. The amount of IMCT in different muscles did not logically relate with the changes in shear force.

Total collagen is generally regarded as an indicator of connective tissue content (Mitchell et al., 1927). Therefore, it was imperative to compare collagen composition of IMCT and epimysium, as the focus of the latter part of this research was the epimysium. In the muscles studied, about 37.3-46.3% of IMCT was collagen, on a dry weight basis. In other words, more than 50% of the (dry) weight of IMCT was non-collagenous in origin. On the other hand, total protein contents of heifer and cow epimysium were about 38 and 44% on a wet weight basis and about 73.5 and 81.9% on a dry weight basis, respectively. Therefore, non-protein matter had contributed to about 25-35% of the weight of the epimysium. Collagen had contributed to about 90% of

epimysial proteins. Contribution of collagen to the weight of total muscle protein was less than 6% in any muscle studied. Remarkably, the ratio between total collagen and intramuscular connective tissue (37-43 g of collagen in 100 g of IMCT) was constant among muscles and also between maturity groups.

The solubility of collagen in Ringer's solution at 70 ºC was influenced by both muscle (type) and the maturity of animals. Accordingly, collagen from GM and L muscles from heifers was more soluble than collagen from the same muscles from cows. However, the solubility of collagen from BF and SM muscles did not differ between cows and heifers. Soluble collagen content calculated on a total collagen basis had produced slightly different results, collagen from SM, GM and L muscles from heifers were significantly more soluble than that from cows but soluble collagen content of BF muscles was not influenced by the animal maturity. All in all, the reduced solubility of intramuscular collagen is not only related to the maturity of animals but also related to the functional needs of muscles. The solubility of collagen from *longissimus* epimysium in water was studied at four different temperatures, 55, 70, 80 and 95 ºC. The fraction analysed was referred to as 'thermolabile protein'. At 55 and 70 ºC, animal maturity*heating time interaction had significantly influenced the protein liberation. At corresponding heating times longer than 60 min at 55 ºC, heifer epimysium had released considerably higher amounts of protein than cow epimysium. After 60 min heating at 70 ºC, heifer epimysium had released about 2.3±0.9% proteins whereas, cow epimysium had released 0.78±0.5% protein, on a weight basis. Temperature increase from 55 to 70 ºC had a clear effect on the amount of protein released from heifer epimysium and also the speed of the process; for instance, about 5.6±1.6% heifer epimysial proteins (on a weight basis) were released after 540 min heating at 55 ºC. Similar amount of protein was released from heifer epimysium after 180 min heating at 70 ºC. During the short heating times employed at 80 and 95 ºC (a maximum of 60 min), the effect of animal maturity was eliminated and epimysium from cows and heifers had

released similar amounts of protein. However, more proteins were released from epimysium after heating at 95 ºC (2.1±1.6) than at 80 ºC (0.87±0.6). Thus, it is clear that the thermolabile protein (collagen) release in an aqueous media is subjected to the combined effects of heating time and temperature (up to 95 ºC); the higher the temperature the lower the time required to release the same amount of protein and vice versa. Therefore, cooking connective tissue rich meat at a high temperature for a short time would be advantageous to obtain the optimum solubility of collagen.

The solubility of collagen in acids and alkali was also investigated at 55 and 70 ºC after pre-equilibration treatments, for 0, 90 and 180 min. These, relatively short, pre-equilibration treatments were selected to match with the high and low concentrations of acids and alkali; because it was noted in preliminary experiments that changes were faster in high concentrations than at low concentrations. Strong (HCl) and weak (CH_3COOH) acids had different effects on epimysial protein solubility. The interaction acid concentration*temperature had influenced the protein release after HCl treatment. Also, it was noted that after 90 min pre-equilibration in HCl epimysium had released more proteins than samples that did not receive a pre-equilibration treatment. The concentration of CH_3COOH had no effect on protein release. Instead, the length of the pre-equilibration treatment and temperature of heating had increased protein liberation after CH_3COOH treatment. This observation is not surprising because, strongly dissociating HCl had a ready supply of H^+ ions to act on proteins whereas dissociation of acetic acid is limited as explained by *Ka* ($1.74x10^{-5}$). Thus, the release of H^+ ions from CH_3COOH is related to the removal of H^+ ions from the medium that is through binding to biomolecules. Therefore, longer pre-equilibration times had favoured the dissociation of CH_3COOH and in turn protein release. After 0.5 M HCl treatment at 70 ºC, about 10% of cow epimysial proteins were released and which is several folds higher than the amounts released with water at the same temperature. Protein release after two alkali treatments, NaOH and NH_4OH, was controlled by

the same factors, pre-equilibration time and the concentration of alkali where temperature of heating had no effect. Within the concentrations employed, (NaOH concentrations were ten times weaker than that of NH_4OH) the two alkalis had released similar amounts of proteins, proving the strong action of a completely dissociating alkali, NaOH. Except for 0.5 M concentration, no other concentration of HCl, CH_3COOH and NH_4OH was superior to water in their ability to release epimysial proteins without a pre-equilibration treatment.

When the epimysium was treated with 0.5 M HCl and subsequently heated to 70 ºC, about 23±10% of its proteins (on a protein basis) were released and therefore, about 77% of proteins were retained in the epimysial residue but their degree of denaturation was unknown. After heating to 70 ºC with 0.5 M CH_3COOH, about 3.4±1.4% of epimysial proteins (on a protein basis) were released retaining ~97% of proteins in the residue. Conversly, shear stress of epimysium was considerably decreased (compared to that of raw epimysium) under the same treatments listed above; the lowest shear stress values for HCl and CH_3COOH treated epimysium were 1.8±1.1 N/mm^2 and 5±4 N/mm^2, respectively. A similar observation was made with 0.05 M NaOH treatment at 70 ºC, where only about 4±2.8% epimysial proteins were released and thus, ~96% of its proteins were retained. However, under the same conditions, shear stress was significantly reduced to 3±1.5 N/mm^2. Therefore, it is thought that proteins retained in the epimysial residue were highly altered and contributed to the decreasing shear stress values.

The collagen that had undergone a phase transition, amorphous collagen, was extracted from heat-treated epimysium using pronase. This fraction of protein was designated as 'pronase liberated protein'. Within the temperature range of 55-95 ºC, the amount of collagen that became amorphous was not influenced by the maturity of animals. The amount of amorphous collagen extracted from epimysium was increased with the length of the heating time at 55 ºC.

As the temperature was increased to 70 ºC, heating time had no statistically significant effect (P=0.383) on amorphous collagen produced, however visual observation of data showed a decrease. Proving the previous observation, amorphous collagen content was significantly decreased with long heating times at 80 and 95 ºC. For example, 5 min heating at 80 or 95 ºC had released about 15.9±2% as amorphous collagen (on a weight basis) but increasing heating time to 60 min had decreased the amount to 14.5±1.5%. As observed at 70, 80 and 95 ºC, a short exposure (≥5 min) to these temperatures had apparently completed the phase transition of epimysial collagen (14-16%) on a weight basis. Similar amounts of amorphous collagen were still not formed after 24 h heating at 55 ºC (8±4% on a weight basis).

According to some of the earlier work, denatured collagen has the ability to recoil and the phenomenon is called 'reversible denaturation'. Also, it is likely that pronase extracts only the collagen that is irreversibly denatured and lost the ability to recoil (Hörmann and Schlebusch, 1971). If these statements are true, amorphous collagen extracted in this experiment represented only the irreversibly denatured collagen as some renaturation likely had occurred. Based on these findings, several conclusions were made; (a) the amount of (irreversibly denatured) amorphous collagen extracted by pronase has an upper limit. (b) The higher the temperature of heating the faster the phase transition occurs. This transition is completed when the right energy requirement is met and which is defined in these experiments as temperature/time combination. (c) The amount of amorphous collagen produced and extracted by pronase was independent from maturity of the animals (heifers of <30 months age and cows of 3-5 years age). However, the hypothesis set, 'the degree of conversion of collagen from a native state to an amorphous state determines the degree of connective tissue driven toughness' (measured as shear stress), was rejected because shear stress of epimysium had continued to decrease with increasing heating time at all the temperatures tested while amorphous collagen had reached a status quo at 70 ºC and above.

The total amount of collagen extracted as thermolabile and amorphous (on a protein basis) was a good indicator of the amounts of protein (considered) completely denatured. When 60 min heating was considered as a reference point for comparison, it is clear that at 55 ºC about 15±4% of heifer epmysial proteins and 12.6±8% of cow epimysial proteins were irreversibly denatured (thermolabile + pronase liberated proteins). As the temperature was increased to 70 ºC, a total of about 40±10% of epimysial proteins were irreversibly denatured (maturity of animal had no effect). As the temperature was further increased to 80 and 95 ºC, total proteins extracted (on a protein basis) was influenced by the interaction, maturity*temperature. However, considering the high standard deviations observed it was thought that cow and heifer epimysium had produced similar amounts of irreversibly denatured collagen (37-46%) after 80 and 95 ºC heat treatments or to that matter retained similar amounts of heat resistant proteins. Under the same circumstances, at 80 and 95 ºC, shear stress of cow epimysium (6.5±2.2 N/mm^2) was significantly higher than that of heifer epimysium (2.8±0.7 N/mm^2) indicating that amounts of protein denatured and/or amounts of protein retained as heat resistant had no logical relationship to shear stress values observed.

The determination of EC and pyrrolic cross-links had helped to understand the response of cross-links to heating. EC was liberated with amorphous collagen but the amount of EC (expressed as units of absorbance) present in a unit weight of amorphous collagen was a constant. Thus, it was hypothesised that EC is uniformly distributed in thermolabile areas of collagen molecule that undergoes phase transition. EC content/g of amorphous collagen did not vary between two maturity groups. Pyridinoline cross-link content/g of protein was related to the maturity of animals before and after heating; cow epimysium always had more cross-links than heifer epimysium. Pyridinoline cross-links were released during aqueous heating at 70 ºC and thus it was thought that pyridinoline cross-links are located in heat sensitive regions of

collagen molecules. Bailey and Lister (1968) had first proposed that some covalent intermolacular cross-links of tendon collagen could be thermolabile and ruptured during thermal shrinkage because tendon lost tension during heating. However, the cross-links involved were not identified.

The thermal denaturation of meat and epimysial proteins was studied using DSC thermograms. The bonds requiring low levels of energy for denaturation must have undergone changes before the peak temperature is reached or in other words just after the onset of denaturation. The bonds requiring high levels of energy would continue to absorb heat and (perhaps) as their numbers are low, the thermogram moves upward from the peak. The DSC thermograms of meat had shown denaturation temperature peaks typical to meat proteins and they were within the following temperature ranges; peak I at 52.4-52.8 ºC, peak II at 60.8-62.9 ºC and peak III at 72.1-72.8 ºC. Among them, the peak II was attributed to collagen due to two reasons; isolated IMCT from the same muscles had produced one clean peak at 58.8-61.8 ºC and it was also noted that 90% of epimysial proteins are collagen. The observations of the present study matches the earlier findings of Martens et al. (1982), who proposed that thermal transition of myosin, collagen and actin occurs within 40-60 °C, 56-62 °C and 66-73 °C, respectively.

The denaturation of IMCT collagen began at 51.1-55.8 °C and ended at 67.2-70.9 °C. Therefore, the temperature selection for the next phase of the research was based on these findings to include 55 and 70 ºC, which cover the phase transition of collagen. The thermal denaturation temperature of cow epimysial collagen (63.02±1.85 °C**)** was similar to that of IMCT. The phase transition onset temperature observed at 55 ºC was confirmed by the scanning electron micrographs and pronase liberated protein data. Further, collagen phase transition was ended at ~80 °C according to pronase released protein data and DSC thermograms for epimysial

proteins. It was imperative to note that DSC experiments were conducted when IMCT and epimysium samples were saturated in water because according to Rochdi et al. (1999) thermal denaturation temperature was dependant on the moisture content of the sample; the higher the moisture the lower the thermal denaturation temperature was.

The narrow temperature range observed for DSC thermograms of IMCT might be resulting from the elimination of matrix effect of meat during the isolation process. In light of the present study, it is important to revisit the proposal from Wright and Humphrey (2002). According to them, the phase transition temperature of collagen is not a specific, 'thermodynamically defined temperature' for the denaturation of collagen but the same degree of denaturation (phase transition) of collagen can be achieved through different combinations of temperature and rates of heating'. In the present study, the maximum amount of amorphous collagen (14-16%) was produced after 5 min exposure to temperatures above 70 ºC. Even though the same amount of amorphous collagen, as at 70 ºC, was not produced after 24 h heating at 55 ºC, gradually increasing amounts of amorphous collagen was made with the increasing heating time. This was a sure sign that 'the same degree of denaturation of collagen can be achieved through different combinations of temperature and rates of heating' as suggested by Wright and Humphrey (2002).

Toughness of meat was mechanically determined and presented as shear force (N) without correcting for the cross-sectional area. For the comparison of shear force from muscles cooked to 71 ºC, data were segregated as the maximum shear force, connective tissue driven shear force and myofibrillar protein driven shear force. The maximum shear force of GM and L muscles were originating from myofibrils and that of BF muscles were originating from the connective tissues. The shear force maximum of SM muscles from cows was equally contributed by the myofibrils and connective tissues, whereas that of heifers was contributed by the myofibrils.

Also, the highest values for myofibrillar and connective tissue driven shear force were observed for BF muscles from cows. The lowest values for myofibrillar and connective tissue driven shear force were observed for L muscles from heifers. Within a muscle, meat from cows had significantly higher shear force maximum values than meat from heifers, except for GM. Shear force of GM was measured after the removal of a distinct band of connective tissue and which is a common practice.

The force required to cut through a piece of epimysium was measured and presented as shear stress, that is the force applied on a unit cross-sectional area of epimysium. The shear stress values for raw epimysium from heifers and cows were about 30.8±3 and 39.6±6.6 N/mm^2. After heating at 55, 70, 80 and 95 ºC shear stress was decreased with the increasing heating time. Despite the previous observation, the shear stress values observed after 1 h heating at 55 ºC (21±8 N/mm^2) was considerably higher than the shear stress values observed after 5 min heating at 70 ºC (8.6±7.5 N/mm^2). This shows that, the higher the temperature the faster the shear stress decrease was. Shear stress was continued to decrease with the increasing heating time at 80 or 95 ºC, for example, after 60 min heating at 80 or 95 ºC shear stress was decreased to 3.6±2.8 N/mm^2. As seen from the above data, the temperature increase had a significant effect on shear stress decrease of epimysium. Most importantly, when heated in an aqueous medium, shear stress of cow epimysium remained higher than that of heifer epimysium at all temperature treatments investigated.

Shear stress values of epimysium after acid and alkali treatments were also measured. From the two acids used, HCl had a clear effect on epimysial shear stress reduction. The combined effect of increasing pre-equilibration time in HCl and temperature of heating had considerably reduced the shear stress values. For example, pre-equilibration in HCl for 180 min and subsequent heating to 70 ºC had decreased the shear stress to 1.8±1.1 N/mm^2 and meet the goal set in this

experiment, to decrease shear stress <2.5 N/mm^2. In addition, also the increasing concentration of HCl had decreased the shear stress values. The combined effect of pre-equilibration time in CH_3COOH and temperature of heating had decreased the shear stress values of CH_3COOH treated epimysium. In general, at matching treatments, shear stress of CH_3COOH treated epimysium was higher than that of HCl treated epimysium. The lowest shear stress values (5±4 N/mm^2) for CH_3COOH treated epimysium were observed after 180 min pre-equilibration in acid together with heating to 70 ºC and this was a considerable reduction compared the shear stress values of raw epimysium (38±7 N/mm^2). Pre-equilibration of epimysium in 0.25 M HCl for a relatively short time (3 h) and subsequent heating to 70 ºC did not produce clear signs of protein denaturation on IR spectra. Therefore, it was thought that the shear stress reduction observed after short pre-equilibration times in HCl (perhaps for CH_3COOH as well) was resulting from the neutralization of charges on proteins and subsequent loss of protein-protein attraction. Fragmentation of proteins to peptides by HCl was thought to be low under these circumstances. However, after overnight pre-equilibration of epimysium in 0.25 M HCl (and CH_3COOH), no denaturation peaks were observed on DSC thermograms. Thus, overnight pre-equilibration in 0.25 M HCl might have produced peptides to completely denature epimysial proteins. Also, the very low shear stress values observed after 0.5 M HCl treatment might be resulting from H^+ mediated peptide cutting because the H^+ concentration was high.

After NaOH treatment, a considerable reduction in stress was noted due to the combined effect of increasing alkali concentration and temperature. For example, heating with 0.05 M NaOH at 70 ºC had decreased shear stress to 3±1.5 N/mm^2. It was unexpected to have high shear stress values (39±3.5 N/mm^2) for NH_4OH treated epimysium at 55 ºC. However, the high thermal denaturation temperature of collagen after NH_4OH treatment (66 ºC) had verified the epimysial stabilizing effect of the alkali. According to Herbine and Dyke (1985) and Nelson et al. (1987) NH_3 and H_2O does not form an ionic complex as expected in $NH_4^+ OH^-$ but stay as a NH_3.H2O

complex and has strong hydrogen bonding properties. Therefore, the observed high shear force values at 55 ºC (similar to raw shear force values) might be resulting from the strong hydrogen bonds between NH_3 and epimysial proteins, which were not destroyed after heating at 55 ºC. For NH_4OH treated epimysium, neither alkali concentration nor pre-equilibration time had any effect on shear force reduction but temperature increase to 70 ºC had significantly decreased shear stress values (5±2.6 N/mm^2).

It is necessary to understand the contribution of thermostable proteins, retained in the epimysium after aqueous heating, to shear stress. As discussed, at 80 or 95 ºC, about 37 and 46% cow and heifer epimysial proteins were denatured (thermolabile + pronase released proteins), respectively. Thus, 54-63% of proteins retained in epimysium were non amorphous and non thermolabile and their degree of denaturation was unknown. It is also imperative to note that cow epimysium had retained more pyridinoline cross-links (56.2±15.3 n moles/g of protein) in the residue after heating for 3 h at 70 ºC than heifer epimysium (16.5±2.0 n moles/g of protein). It was thought, that the high amounts of protein and pyridinoline cross-links retained in epimysium might have contributed to the observed high shear stress values of cow epimysium after heating to 80 and 95 ºC.

Shrinking of epimysium was measured during heating at 80 and 95 ºC, where cow epimysium shrank more in length than heifer epimysium, 68±6% and 46±10%, respectively. In contrast, cow epimysium contained more pyridinoline cross-links than heifer epimysium (before and) after heating at 70 ºC. Thus, it could be hypothesised that epimysial collagen shrinkage was an independent phenomenon not influenced by cross-linking because cow epimyisum contained more pyridinoline cross-links than heifer epimysium after heating. As mentioned already, Bailey and Lister (1968) thought that some (unidentified) cross-links of collagen could be disintegrated during thermal shrinkage. However, the true cause for the difference between cow

and heifer epimysial shrinkage was not elucidated in this study. Wall et al. (1999) had shown that shrinkage is very rapid at high temperatures (62-65 ºC) but slow at long exposure to low temperatures. The shrinkage could be as fast as a few seconds to complete the process and followed a sigmoid pattern (Chen et al., 1997; Chen et al., 1998). These authors had also shown that collagen shrinkage should be measured while heating because during cooling the tissue could recover. Perhaps, these may be the reasons for the lack of an effect from heating time, and temperatures in the present study. Collagen shrinkage is thought to be resulting from the helix to coil transition (Lepetit, 2008). In the present study, no apparent relationship can be observed between shrinkage and other measures of denaturation such as thermolabile protein or amorphous collagen.

Changes in epimysium thickness subsequent to heating was investigated and which was unidirectional measurement related to swelling of collagen. According to literature, this is the first attempt to relate epimysial thickness change with heat induced collagen denaturation. Epimysial thickness change was not influenced by the maturity of animals except at 70 ºC. At the two (relatively) low temperature treatments, 55 and 70 ºC, thickness increased as heating time was increased but when the temperature was further increased to 80 and 95 ºC, neither temperature nor heating time had any effect on thickness. It is important to mention that standard deviations for thickness were very high and thickness values of points on epimysium 2 cm apart were significantly different after heating. This may be resulting from the inherent variability of the tissue due to uneven distribution of capillaries, nervous tissue (and so on) or in other words the matrix effect; the study is still significant as a 'phenomenological model' (Wright and Humphrey, 2002). The epimysial thickness increase after aqueous heating was thought to be resulting from the heat induced rupture of tissues and subsequent entry of water to bind to proteins. The degree of increase in epimysial thickness was decreased as a result of the combined effect of increasing HCl concentration, pre-equilibration time and temperature;

thought to be resulting from protein hydrolysis and release. Maybe due to significantly low protein release after CH_3COOH, NaOH and HH_4OH treatments, epimysial thickness was increased with increasing concentration, pre-equilibration time and temperature (as combined or individual effects of one or many of them). Importantly, epimysial thickness increase after NaOH and HH_4OH treatments at 55 and 70 ºC was similar to that of water but acids act differently; HCl decreased the thickness and CH_3COOH increased the thickness more than water at the same temperature.

Water absorption in terms of epimysial weight gain during heating was investigated and it followed a trend similar to that of thickness change at 55 and 70 ºC. At the same temperatures, epimysial weight gain was not related to the maturity of animals but increased with the increasing temperature and heating time. The highest values for weight gain were reported at 70 ºC, between corresponding heating times. As the temperature was increased to 80 and 95 ºC, weight gain was gradually decreased compared to the values at 70 ºC and also the effect of heating time was eliminated. However, heifer epimysium held more water at 95 ºC (47.5±7%) than that at 80 ºC (32.6±12%). Also, heifer epimysium held more water than cow epimysium at either of the temperatures (38±4%). It is thought that water uptake is closely related to protein denaturation as it increased with the increasing energy supply at 70 ºC. However, a clear relationship was not observed between two parameters; for example, after 360 min heating at 70 ºC, weight gain had reached the highest value of 80±26% (continually increased with the heating time) while amorphous collagen (denatured protein retained in epimysium) was 12±3% on a weight basis (the latter was at a status quo during all heating times). As acids and alkali act on covalent peptide bonds of protein and release peptides, it was expected to have decreased water absorption after strong acid and alkali treatments. However, a decrease in water uptake was noted only after HCl treatment combined with heating time and temperature. At the concentrations used and subjected to the other conditions of the experiment CH_3COOH, NaOH

and NH_4OH had increased the epimysial water uptake. After 180 min pre-equilibration in 0.5 M CH_3COOH, epimysium gained the highest weight (out of 4 acid and alkali treatments) of 134±39%. Epimysium had gained more weight after heating in CH_3COOH than heating in water. In general, water uptake was higher with NaOH than with NH_4OH; for example, after 180 min pre-equilibration in 0.05 M NaOH epimysial weight gain was about 73±8% but after 0.5 M NH_4OH treatment, weight gain was 46.7±13%. Low values of water uptake for NH_4OH treated epimysium may be resulting from observed (already discussed under thermal denaturation temperatures) structure stabilization effect of NH_4OH.

It is imperative to discuss the some of the epimysial properties that lead to the high standard deviations observed in results in general and specifically in the last study. It was observed during these studies that thickness of epimysium decreased outwards from the centre of the body; thickness variation was in the range of about <3.0 to <0.5 mm. From anterior to posterior of the animal a gradual decline in epimysial thickness was observed, however, this was not as significant as the lateral variation. Epimysium from cows was thinner than those from heifers and the latter contained more fat. Thickness is directly related to the weight and evidently all the other properties of epimysium. Systematic sampling was carried out during the second phase of the experiment where an epimysial strips was removed along the length and a single temperature treatment was allocated to a strip to minimize the effect of dramatic drop of thickness within a treatment. However sampling methodology was changed during the last phase of the study to eliminate the location specific effect. This lead to a high degree of variation in thickness (thereby other properties) in samples exposed to the same treatment resulting the high standard deviations. This clearly demonstrates the difficulty in working with intact animal tissue.

7. GENERAL CONCLUSIONS

Based on the results of the first study, it was concluded that the amount of intramuscular connective tissue present in a muscle was a feature unique to the muscle type (designated as *biceps femoris*, *semimembranosus*, *gluteus medius* and *longissimus)* and which was influenced by the maturity of cattle (cows of 6±1 years and heifer of 16±2 months). It was also concluded that the ratio of total collagen to intramuscular connective tissue was a constant across muscles studied and also a constant between cows and heifers and therefore, total collagen was a good indicator of intramuscular connective tissue content of these muscles. Also, lack of a rational link between (maximum) shear force and intramuscular connective tissue content had lead to the conclusion that the shear force of meat could not be predicted by the quantity of intramuscular connective tissue. Soluble collagen of meat was not a predictor of shear force changes in muscles. The general trend observed that collagen from cow meat was less soluble than that from heifers was contradicted when BF from cows and heifers had similar amounts of soluble collagen. This had lead to the conclusion that collagen solubility/insolubility was not simply an age dependant factor but was dependant on the functional needs of muscles. It was also concluded that, peak II of DSC thermograms for meat originated from intramuscular connective tissue proteins.

According to the observations of the second study, it was concluded that a brief exposure (about 5 min) to temperatures between 70 and 95 ºC could convert the maximum possible amount of collagen to an amorphous state and which was independent from the maturity of cattle. The same degree of change in shear stress decrease, weight gain, thickness increase, thermolabile and amorphous protein release could be attained by providing similar input of energy as

depicted by different combinations of temperatures and heating times. Because Ehrlich chromogen were detected in amorphous collagen and pyridinoline cross-links were released after extended heating, it was concluded that at least some of these cross-links were located in heat sensitive areas of collagen molecules. Because the ratio between EC (absorbance units at 572 nm) and the weight of amorphous collagen (in g) was a constant for cow and heifers and also a constant for all combinations of heat treatments, it was concluded that EC was evenly distributed in areas of collagen molecule that could become amorphous.

Significant amounts of proteins were retained in the epimysium after 0.25 M HCl treatment. IR spectra were mostly unchanged for amide I and II bands after the same HCl treatment. However, shear stress was considerably decreased under the same circumstances. Therefore, it is hypothesised that, under the conditions of the experiment, decreased shear stress of epimysium may be due to weakened epimysial structure resulting from H^+/Cl^- mediated neutralization of charged ions on protein surfaces and consequential lack of attraction between proteins. It was concluded that the shear stress decreasing ability of 0.1, 0.25 and 0.5 M HCl, though a strong acid, was similar to that of 0.1, 0.25 and 0.5 M CH_3COOH at low temperatures (55 ºC) and at relatively low pre-equilibration times (180 min). Based on the increased thermal denaturation temperature of NH_4OH treated epimysium and also based on the high shear stress values observed for NH_4OH treated epimysium after heating at 55 ºC, it was concluded that NH_4OH had an epimysium stabilization effect which could not be eliminated by heating at 55 ºC.

8. FUTURE DIRECTIONS

- Pyridinoline, being a trivalent and a mature form of cross-link, could be one of the contributors to the observed higher shear stress values of cow epimysium. Therefore, it is proposed to carry out further investigation on the thermal properties of pyridinoline cross-links (cross-links retained in a unit volume of meat or connective tissue), sensitivity of pyridinoline to acid and alkali treatments and to identify the location of pyridinoline on the collagen molecule.

- The work presented in this thesis did not assess the contribution of proteoglycans to the shear stress of epimysium. It was proposed that glycosaminoglycans were bound to type I collagen through electrostatic bonds (Scott, 1988). However, no investigations were carried out to elucidate the effect of proteoglycans on shear stress of connective tissues. Therefore, future research could be focused on the types of proteoglycans in the epimysium and their relationship to shear stress.

- Theoretically, swelling of collagen was related to the number of cross-links present in a unit volume of collagen fibres and thus the higher the cross-link content per volume was the less the swelling was (Lepetit, 2008). It was reported in this thesis that cow epimysium contained higher amounts of pyridinoline (trivalent) cross-links than heifer epimysium before and after heating but EC (trivalent) cross-link content in (at least) amorphous areas of collagen (after a heat treatment) did not differ between cows and heifers. (Note that pyridinoline and EC were presented on a weight basis). Therefore, it would be of interest to establish the actual relatonship between (type of) cross-link content and swelling properties.

- An experiment similar to the reported experiment with acids and alkalis could be conducted using epimysium/connective tissue but with low concentrations and with a wide range of pre-equilibration treatments (varying from short to very long times) to facilitate the asymptotic equilibration. This would help to establish reasonable concentrations of acids and alkalis for industrial applications.

- Shrinkage of isolated collagen fibres was completed within seconds from reaching the optimum temperature for the transformation (Chen et al., 1997). At the same time shrinkage of epimysium/connective tissue was not investigated other than in the present study but in a minutes (time) scale. Therefore, it would be of interest to understand the similarities and differences between shrinkage of epimysium/connective tissue and isolated collagen (from epimysium) in a seconds (time) scale. Such an experiment would be planned using cattle from a range of age groups, for example, post natal to very old animals (>20 years).

- The possibility of renaturation of heat-denatured collagen was reported in the literature (Hörmann and Schlebusch, 1971). The present experiments did not account for the possibilities of renaturation of collagen after heating. Therefore, it would be of interest to quantify the helix to coil transformation of both matrix-bound and isolated collagen prior to renaturation (during cooling). Identification of an analytical method to measure an ongoing transformation would be critical in this study.

- As the properties of collagen and its types can be influenced by the animal nutrition and number of parturitions, it is important to investigate their impact on toughness of connective tissues.

9. REFERENCES

Abbott, M. T., Pearson, A. M., Price, J. F., & Hooper, G. R. (1977). Ultrastructural changes during autolysis of red and white porcine muscles. *Journal of Food Science, 42*, 1185-1188.

Adams, P., Eyre, D. R., & Muir, H. (1977). Biochemical aspects of development and ageing of human lumber intervertebral discs. *Rheumatology and Rehabilitation, 16*, 22-29.

Aktas, N., & Kaya, M. (2001). The influence of marinating with weak organic acids and salts on the intramuscular connective tissue and sensory properties of beef. *European Food Research and Technology, 213*, 88-94.

Aktas, N., & Kaya, M. (2001). Influence of weak organic acids and salts on the denaturation characteristics of intramuscular connective tissue. A differential scanning calorimetry study. *Meat Science, 58*, 413-419.

Aktas, N., & Aksu, M. I., & Kaya, M. (2003). The effect of organic acid marination on tenderness, cooking loss and bound water content of beef. *Journal of Muscle Foods, 14*, 181-194.

Alsmeyer, R. H., Thornton, J. W., & Hiner, R. L. (1965). Some dorsal-lateral location tenderness differences in the longissimus dorsi muscle of beef and pork. *Journal of Animal Science, 24*, 526-530.

Andreassen, T. T., Seyer-Hansen, K., & Bailey, A. J. (1981). Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. *Biochemica et Biophysica Acta, 677* (2), 313-317.

Anna, A. M., Lakshmanan, V., Radha, K., Singh, R. P., Mendiratta, S. K., & Anjanevulu, A. S. R. (2007). Effect of blade tenderization and sodium bicarbonate on quality of buffalo rumen meat. *Journal of Food Science and Technology (Mysore), 44* (4), 437-439.

Arganosa, G. C., & Marriott, N. G. (1989). Organic acids as tenderizes of collagen in restructured beef. *Journal of Food Science, 54* (50), 1173-1176.

AOAC. *Official Methods of Analysis,* 17th ed.; Association of Official Analytical Chemists: Gaithersburg. Washington, D. C., 1990.

Avery, N. C., & Bailey, A. J. (1995). An efficient method for the isolation of intramuscular collagen. *Meat Science, 41* (1), 97-100.

Avery, N. C., & Bailey, A. J. Restraining cross-links responsible for the mechanical properties of collagen fibres: Natural and artificial. In *Collagen structure and mechanics;* Fratzl, P., Ed.; Springer: 2008, pp 81-110.

Avery, N. C., Sims, T. J., Warkup, C., & Bailey, A. J. (1996). Collagen cross-linking in porcine m.longissimus lumborum: absence of a relationship with variation in texture at pork weight. *Meat Science, 42* (3), 355-369.

Bächinger, H. P. (1967). The influence of peptidyl-prolyl cis-trans isomerase on the in vitro folding of type III collagen. *The Journal of Biological Chemistry. 262* (35), 17144-17148.

Bailey, K. (1948). Tropomyosin: a new asymmetric protein component of the muscle fibril. *The Biochemical Journal, 43* (2), 271-279.

Bailey, A. J., & Lister, D. (1968). Thermally labile cross-links in native collagen. *Nature, 220*, 280-281.

Bailey, A. J., & Peach, C. M. (1968). Isolation and structural identification of a labile intermolecular crosslink in collagen. *Biochemical and Biophysical Research communications, 33,* 812-819.

Bailey, A., J. & Sims, T. T. (1977). Meat tenderness: distribution of molecular species of collagen in bovine muscle. *Journal of the Science of Food and Agriculture, 28* (6), 565-570.

Bailey, A. J. (1985). The role of collagen in the development of muscle and its relationship to eating quality. *Journal of Animal Science, 60*, 1580-1587.

Bailey, A. (1989). The chemistry of collagen cross-links and their role in meat texture. *Proceedings of the Reciprocal Meat Conference*, 127-135.

Bailey, A. J., Paul, R. G., & Knott, L. (1998). Mechanisms of maturation and ageing of collagen. *Mechanisms of Ageing and Development, 106*, 1-56.

Ball, C. O., Clauss, H. E., & Stier, E. F. (1957). Factors affecting the quality of prepackaged meat. IB. Loss of weight and study of texture. *Food Technology, 11*, 281-284.

Batzer, H., & Kreibich, T. (1981). Influence of water on thermal transitions in natural polymers and synthetic polyamides. *Polymer bulletin, 5*, 585-590.

Bayne, B. H., Meyer, B.H., & Cole, J. W. (1969). Response of beef roasts differing in finish, location and size to two rates of heat application. *Journal of Animal Science, 29*, 283-287.

Bayne, B. H., Strawn, S. S., Hutton, C. W., Backus, W. R., & Bernadine, H. (1971). Relationship of alkali and ringer insoluble collagen to tenderness of beef heated at two rates. *Journal of Animal Science, 33,* 958-96.

Baronas-Lowell, D., Lauer-Fields, J. L. & Fields, G. B. (2003). Defining the roles of collagen and collagen like proteins within the proteome. *Journal of Liquid Chromatography, 26* (14), 2225-2254.

Baum, J., & Brodsky, B. (1999). Folding of peptide models of collagen and misfolding in disease. *Current Opinion in Structural Biology, 9* (1), 122-128.

Bax, D. V., Bernard, S. E., Lomas, A., Morgans, A., Humphries, J., Shuttleworth, C. A., Humphries, M. J., & Kielty, C. M. (2003). Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. *The Journal of Biological chemistry, 278* (36), 34605-34616.

Bechtel, P. J., & Parrish, F. C. (1983). Effects of postmortem storage and temperature on muscle protein degradation: Analysis by SDS gel electrophoresis. *Journal of Food Science, 48*, 294-295.

Behrends, J. M., Goodson, K. J., Koohmaraie, M., Shackelford, S. D., Wheeler, T. L., Morgan, W. W., Reagan, J. O., Gwartney, B. L., Wise, J. W., & Savell, J. W. (2005). Beef customer satisfaction: Factors affecting consumer evaluations of calcium chloride-injected top sirloin steaks when given instructions for preparation. *Journal of Animal Science, 83*, 2869-2875.

Bell, L. N., & Hageman, M. J. (1996). Glass transition explanation for the effect of polyhydroxy compounds on protein denaturation in dehydrated solids. *Journal of Food Science, 61* (2), 372-375.

Bella, J., Eaton, M., Brodsky, B., & Berman, H. M. (1994). Crystal and molecular structure of a collagen-like peptide at 1.9 Å resolution. *Science, 266* (5182), 75-81.

Bella, J., & Berman, H. M. (1996). Crystallographic evidence for C^{α}-H…O=C hydrogen bonds in a collagen triple helix. *Journal of Molecular Biology, 264*, 734-742.

Bendall, J. R., & Restall, D. J. (1983). The cooking of single myofibres, small myofibre bundles and muscle strips from beef *M. psoas* and *M. sternomadibularis* muscles at varying heating rayes and temperatures. *Meat science, 8*, 93-117.

Benson, J. R., & Hare, P. E. (1975). O-Phthalaldehyde: Fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proceedings from the National Academy of Sciences USA,72* (2), 619-622.

Bentley, J. P. (1979). Aging of collagen. *The Journal of Investigative Dermatology, 73*, 80-83.

Berg, R. A., Schwartz, M. L., Rome, L. H., & Crystal, R. G. (1984). Lysosomal function in the degradation of defective collagen in cultured lung fibroblasts. *Biochemistry, 23* (10), 2134-2138.

Berg, R. A., & Prockop, D. J. (1973). The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. *Biochemical and Biophysical Research communications, 52* (1), 115-120.

Berge, P., Ertbjerg, P., Larsen, L. M., Astruc, T., Vignon, X., & Møller, A. J. (2001). Tenderization of beef by lactic acid injected at different times post mortem. *Meat science, 57*, 347-357.

Berge, P., Ertbjerg, P., Larsen, L. M., Astruc, T., Vignon, X., & Møller, A. J. (2001). Tenderization of beef by lactic acid injected at different times post mortem. *Meat Science, 57*, 347-357.

Bernal, V. M., & Stanley, D. W. (1987). Stability of bovine muscle connective tissues. *Journal of Food Science, 52* (4), 876-878.

Bertram, H. C., Kohler, A., Böcker, U., Ofstad, R., Andersen, H. J. (2006). Heat-induced changes in myofibrillar protein structures and myowater of two pork qualities. A combined FT-IR spectroscopy and low-field NMR relaxometry study. *Journal of Agricultural and Food Chemistry*, 54, 1740-1746.

Biliaderis, C. G., Maurice, T. J., & Vose, J. R. (1980). Starch gelatinization phenomena studied by differential scanning calorimetry. *Journal of Food Science*, 45 (6), 1669-1674.

Boame, A., Parsons, W., & Trant, M. (2004). *Mad Cow Disease and Beef Trade: An Update*. Statistics Canada. http://www.statcan.ca/english/research/11-621-MIE/2004010/11-621-MIE2004010.pdf. (accessed March 14, 2008).

Boccard, R., Buchter, L., Casteels, E., Cosentino, E., Dransfield, E., Hood, D. E., Joseph, R. L., MacDougall, D. B., Rhodes, D. N., Schon, I., Tinbergen, B. J., & Touraille, C. (1981). Procedures for measuring meat quality characteristics in beef production experiments. Report of a working group in the Commission of the European Communities (CEC) beef production research programme. *Livestock Production Science, 8*, 385-397.

Böcker, U., Ofstad, R., Wu, Z., Bertram, H. C., G. D. Sockalingum, Manfait, M., Egelandsdal, B., & Kohler, A. (2007). Revealing covariance structures in fourier transform infrared and raman microspectroscopy spectra: A study on pork muscle fibre tissue subjected to different processing parameters. *Applied Spectroscopy*, 61(10), 1032-1039.

Boleman, S. J., Boleman, S. L., Miller, R. K., Taylor, J. F., Cross, H. R., Wheeler, T. L., Koohmararie, M., Shackelford, S. D., Miller, M. F., West, R. L., Johnson, D. D., & Savell, J. W. (1997). Consumer evaluation of beef of known categories of tenderness. *Journal of Animal Science, 75*, 1521-1524.

Boot-Handford, R. P., & Tuckwell, D. S. (2003). Fibrillar collagen: the key to vertebrate evolution? A tale of molecular incest. *Bio Essays, 25*, 142-151.

Bosselmann, A., Möller, C., Steinhart, H., Kirchgessner, M., & Schwarz, F. J. (1995). Pyridinoline cross-links in bovine muscle collagen. *Journal of Food Science, 60* (5), 953-958.

Bourge, T. K., & Caulfield, J. B. (1980). Morphology of connective tissue in skeletal muscle. *Tissue & Cell, 12* (1). 197-207.

Bouton, P. E., & Harris, P. V. (1972). A comparison of some objective methods used to assess meat tenderness. *Journal of Food Science, 37*, 218-221.

Bouton, P. E., & Harris, P. V. (1972). The effects of cooking temperature and time on some mechanical properties of meat. *Journal of Food Science, 37*, 140-144.

Bouton, P. E., Harris, P. V., & Shorthose, W. R. (1976). Factors influencing cooking losses from meat. *Journal of Food Science, 41*, 1092-1095.

Boutten, B., Brazier, M., Morche, N., Morel, A., & Vendeuvre, J. L. (2000). Effects of animal and muscle characteristics on collagen and consequences for ham production. *Meat Science*, 55, 233-238.

Bowes, J. H., & Kenten, R. H. (1948). The effect of alkalis on collagen. *Biochemical Journal, 43*, 358-365.

Bowes, J. H. & Kenten, R. H. (1950). The swelling of collagen in alkaline solutions. *Biochemistry*, 46, 1-8.

Bowes, J. H., Elliott, R. G., & Moss, J. A. (1955). The composition of collagen and acid soluble collagen of bovine skin. *Biochemical Journal, 61* (1), 143-150.

Brady, D. E. (1937). A study of the factors influencing tenderness and texture of beef. *Journal of Animal Science, 1*, 246-250.

Bratzler, L. J. (1949). Determining the tenderness of meat by use of the Warner-Bratzler method. *Reciprocal Meat Conference Proceedings, 2,* 117-121.

Bressan, G. M., & Prockop, D. J. (1977). Synthesis of elastin in aortas from chick embryos. Conversion of newly secreted elastin to cross-linked elastin without apparent proteolysis of the molecule. *Biochemistry, 16* (7), 1406-1412.

Brodsky, B., & Persikov, A. V. (2005). Molecular structure of the collagen triple helix. *Advances in Protein Chemistry, 70*, 301-339.

Brodsky, B., & Ramshaw, J. A. M. (1997). Collagen triple helix structure. *Matrix Biology, 15*, 545-554.

Brooks, J. C., & Savell, J. W. (2004). Perimysium thickness as an indicator of beef tenderness. *Meat Science, 67*, 329-334.

Buehler, M. J. (2008). Molecular architecture of collagen fibrils: a critical length scale for tough fibrils. *Current Applied Physics, 8*, 440-442.

Bulleid, N. J., Dalley, J. A., & Lees, J. F. (1997). The C-peptide domain of procollagen can be replaced with a transmenbrane domain without affecting trimer formation of collagen triple helix folding during biosynthesis. *EMBO Journal, 16* (22), 6694-6701.

Burke, R. M., & Monahan, F. J. (2003). The tenderization of shin beef using citrus juice marinade. *Meat Science, 63*, 161-168.

Burson, D. E., & Hunt, M. C. (1986). Proportion of collagen type I and III in four bovine muscles differing in tenderness. *Journal of Food Science, 51* (1), 51-53.

Byler, D. M., & Susi, H. (1986). Examination of secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers, 25* (3), 469-487.

Caine, W. R., Aalhus, J. L., Best, D. R., Dugan, M. E. R., & Jeremiah, L. E. (2003). Relationship of texture profile analysis and Warner-Bratzler shear force with sensory characteristics of beef rib steaks. *Meat Science, 64*, 333-339.

Calder, G. V., & Barton, T. J. (1971). Actual effects controlling the acidity of carboxylic acids. *Journal of Chemical Education, 48* (5), 338-340.

Califano, A. N., Bertola, N. C., Bevilacqua, A. E., & Zaritzky, N. E. (1997). Effect of processing conditions on the hardness of cooked beef. *Journal of Food Engineering, 34*, 41-54.

Camacho, N. P., West, P., Torzilli, P. A., & Mendelsohn, R. (2001). FTIR microscopic imaging of collagen and proteoglycan in bovine cartilage. *Biopolymers*, 62, 1-8.

Camou, J. P., Marchello, J. A., Thompson, V. F., Mares, S. W., & Goll, D. E. (2007). Effet of postmortem storage on activity of μ- and m-calpain in five bovine muscles. *Journal of Animal Science, 85*, 2670-2681.

Canty, E. G., & Kadler, K. E. (2005). Procollagen trafficking, processing and fibrillogenesis. *Journal of Cell Science, 118,* 1341-1353.

Caporaso, F., Cortavarria, A. L., & Mandigo, R. W. (1978). Effects of post-cooking sample temperature on sensory and shear analysis of beef steaks. *Journal of Food Science, 43*, 839-841.

Carrino, D. A. (1998). Dynamic expression of proteoglycans during skeletal muscle development. *Basic Applied Myology, 8* (2), 95-106.

Carrino, D. A., Önnerfjord, P., Sandy, J. D., Cs-Szabo, G., Scott, P. G., Sorrel, J. M., Heinegård, D., & Caplan, A. I. (2003). Age-related changes in the proteoglycans of human skin. *Journal of Biological Chemistry, 278* (19), 17566-17572.

Chalovich, J. M. (1992). Actin mediated regulation of muscle contraction. *Pharmacology & Therapeutics, 55* (2), 95-148.

Chen, S. S., Wright, N. T., & Humphrey, J. D. (1997). Heat-induced changes in the mechanics of a collagenous tissue: Isotermal free shrinkage. *Journal of Biomechanical Engineering, 119*, 372-378.

Chen, S. S., Wright, N. T., & Humphrey, J. D. (1998). Phenomenological evolution equations for heat-induced shrinkage of a collagenous tissue. *IEEE Transactions on Biomedical Engineering*, 45 (10), 1234-1240.

Cheng, C. S., & Parrish, F. C. (1976). Scanning electron microscopy of bovine muscle: Effect of heating on ultrastructure. *Journal of Food Science, 41*, 1449-1454.

Christensen, M., Purslow, P. P., & Larsen, L. M. (2000). The effect of cooking temperature on mechanical properties of whole meat, single muscle fibres and perimysial connective tissue. *Meat Science, 55*, 301-307.

Church, F. C., Porter, D. H., Catignani, G. L., & Swaisgood, H. E. (1985). An o-phthalaldehyde spectrophotometric assay for proteinases. *Analytical Biochemistry, 146* (2), 343-348.

Commercial Beef New Markets. *Researching tenderization methods for commercial beef.* Beef information Centre, Calgary AB, Canada, 2008. www.canadianbeef.info.

Cover, S., Ritchey, S. J., & Hostetler, R. L. (1962). Tenderness of beef I. The connective tissue component of tenderness. *Journal of Food Science, 27* (5), 469-475.

Cronlund, A. L., & Woychik, J. H. (1987). Solubilization of collagen in restructured beef with collagenases and α-amylase. *Journal of Food Science, 52* (4), 857-860.

Cross, H. R., Stanfield, M. S., & Koch, E. J. (1976). Beef palatability as affected by cooking rate and final internal temperature. *Journal of Animal Science, 43*, 114-121.

Csiszar, K. (2001). Lysyl oxidases: a novel multifunctional amine oxidase family. *Progress in Nucleic Acid Research and Molecular Biology, 70*, 1-32.

Davey, C. L., & Gilbert, K. V. (1975). Temperature dependent cooking toughness in beef. *Journal of the Science of Food and Agriculture, 25* (8), 931-938.

Davies, R. E. (1963). A molecular theory of muscle contraction: calcium-dependant contractions with hydrogen bond formation plus ATP-dependant extensions of part of the myosin-actin cross-bridges. *Nature, 199*, 1068-1074.

DeFremery, D., & Streeter, J. V. (1969). Tenderization of chicken muscle: The stability of alkali-insoluble connective tissue during post-mortem aging. *Journal of Food Science, 34*, 176-180.

DeGroot, J., Verzijl, N., Bank, R. A., Lafeber, F. P. J. G., Bijlsma, J. W. J., & TeKoppele. (1999). Age-related decrease in proteoglycan synthesis of human articular chondrocytes. *Arthritis & Rheumatism, 42* (5), 1003-1009.

DeGroot, J. (2004). The AGE of the matrix: chemistry, consequence and cure. *Current opinion in Pharmacology, 4* (3), 301-305.

Delgado, E. J., Rubio, M. S., Iturbe, F. A., Méndez, R. D., Cassís, L., & Rosiles, R. (2005). Composition and quality of Mexican and imported retail beef in Mexico. *Meat Science, 69*, 465-471.

Destifanis, G., Brugiapaglia, A., Barge, M. T., & Molin, D. E. (2008). Relationship between beef consumer tenderness perception and Warner-Bratzler shear force. *Meat Science, 78* (3), 153-156.

Dyer, D., Dunn, J. A., Thorpe, S. R., Bailie, K. E., Lyons, T. J., McCance, D. R., & Baynes, J. W. (1993). Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *Journal of Clinical Investigation, 91* (6), 2463-2469.

Dikeman, M. E. (1987). Fat reduction in animals and the effects on palatability and consumer acceptance of meat products. *Reciprocal Meat Conference, 40*, 93-101.

Dikeman, M. E., Tuma, H. J., Glimp, H. A., Gregory, K. E., & Allen, D. M. (1972). Evaluation of the tenderometer for predicting bovine muscle tenderness. *Journal of Animal Science, 34* (6), 960-962.

Drake, M. P., Davison, P. F., Bump, S., & Schmitt, F. O. (1966). Action of proteolytic enzymes on tropocollagen and insoluble collagen. *Biochemistry, 5* (1), 301-312.

Dransfield, E., Rhodes, D.N., Nute, G. R., Roberts, T. A., Boccard, R., Touraille, C., Buchter, L., Hood, D. E., Joseph, R. L., Schon, I., Casteels, M., Cosentino, E., & Tinbergen, B. J. (1982). Eating quality of European beef assessed at five research institutes. *Meat Science, 6*, 163-184.

Dransfield, E., Francombe, M. A., & Whelehan, O. P. (1984). Relationship between sensory attributes in cooked meat. *Journal of Texture Studies, 15*, 33-48.

Dreisewerd, K., Rohlfing, A., Spottke, B., Urbanke, C., & Henkel, W. (2004). Characterization of whole fibril-forming collagen proteins of types I, III and V from fetal calf skin by infrared matrix-assisted laser desorption ionization mass spectrometry. *Analytical Chemistry, 76,* 3482-3491.

Elden, H. R. (1958). Rate of swelling of collagen. *Science, 128*, 1624-1625.

Engvall, E., & Ruoslahti, E. (1977). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *International Journal of Cancer, 20*, 1-5.

Engvall, E., Ruoslahti, E., & Miller, E. J. (1978). Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *Journal of Experimental Medicine, 147*, 1584-1595.

Estoe, J. E. (1955). The amino acid composition of mammalian collagen and gelatin. *Biochemical Journal, 61*, 589-600.

Estoe, J. E. Composition of collagen and allied proteins. In *Treatise on Collagen.* ed. G. Ramachandran, G. N., Ed. Academic Press: New York,.1967, Vol 1, pp 1-67.

Eyre, D. R. (1980). Collagen: Molecular diversity in the body's protein scaffold. *Science, 207* (21), 1315-1322.

Eyre, D. R. (1984). Cross-linking in collagen and elastin. *Annual Reviews of Biochemistry, 53*, 717-748.

Fang, S. H., Nishimura, T., & Takahashi, K. (1999). Relationship between development of intramuscular connective tissue and toughness of pork during growth of pigs. *Journal of Animal Science, 77*, 120-130.

Farah, C. S., & Reinach, F. C. (1995). The troponin complex and regulation of muscle contraction. *Federation of American Societies for Experimental Biology Journal, 9*, 755-767.

Ferguson, D. M., Daly, B. L., Gardner, G. E., & Tume, R. K. (2008). Effect of glycogen concentration and form on the response to electrical stimulation and rate of post-mortem glycolysis in ovine muscle. *Meat Science, 78*, 202-210.

Fernández-Martín, E., Otero, L., Solas, M. T., & Sanz, P. D. (2000) Protein denaturation and structural damage during high-pressure-shift freezing of porcine and bovine muscles. *Journal of Food Science, 65* (6), 1002-1008.

Feuz, D. M., Umberger, W. J., Calkins, C. R., & Sitz, B. (2004) U. S. consumer's willingness to pay for flavor and tenderness in steaks as determined with an experimental auction. *Journal of Agricultural and Resource Economics, 29* (3), 501-516.

Field, R. A., Riley, M. L., & Chang, Y. (1969). Epimysial connective tissue scores as related to beef tenderness. *Journal of Food Science, 34*, 514-518.

Field, R. A., Pearson, A. M., & Schweigert, B. S. (1970). Labile collagen from epimysial and intramuscular connective tissue as related to warner-bratzler shear values. *Journal of Agriculture and Food Chemistry, 18* (2), 280-283.

Field, R. A., Pearson, A. M., Magee, W. T., & Merkel, R. A. (1970). Chemical and histological characteristics of the M. longissimus in young bulls selected for tenderness or leanness. *Journal of Animal Science, 30*, 717-721.

Findlay, C. J., Stanley, D. W., & Gullet, E. A. (1986). Thermomechanical properties of beef muscle. *Meat Science, 16* (1), 57-70.

Findlay, C. J., & Barbut, S. (1990). Thermal analysis of meat. In V. R. Harwalker & C. Y. Ma. *Thermal Analysis of Foods*, (pp92-125). New York, Elsevier Science Publishing Co., Inc.

Flory, P. J. (1956). Role of crystallization in polymers and proteins. *Science, 124* (3211), 53-60.

Flory, P. J., & Garret, R. R. (1958). Phase transition in collagen and gelatin systems, *Journal of American Chemical Society, 80* (18), 4836-4845.

Foegeding, E. A., & Larick, D. K. (1986). Tenderization of beef with bacterial collagenase. *Meat Science, 18,* 201-214.

Fogle, D. R., Plimpton, R. F., Ockerman, H. W., Jarenback, L., & Persson, T. (1982). Tenderization of beef: effect of enzyme, enzyme level and cooking method. *Journal of Food Science, 47*, 1113-1118.

Freemont, A. J., & Hoyland, J. A. (2007). Morphology, mechanisms and pathology of musculoskeletal ageing. *Journal of Pathology, 211*, 252-259.

Friess, W., & Lee, G. (1996). Basis thermoanalytical studies of insoluble collagen matrices. *Biomaterials, 17*, 2289-2294.

Frye, E. B., Degenhardt, T. P., Thorpe, S. R., & Baynes, J. W. (1998). Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependant increase in imidazolium cross-links in human lens proteins. *Journal of Biological Chemistry, 273* (30), 18714-18719.

Fujii, K., & Murota, K. (1982). Isolation of skeletal muscle collagen. *Analytical Biochemistry*, *127*, 449-452.

Fujimoto, D., Akiba, K., & Nakamura, N. (1977). Isolation and characterization of a fluorescent material in bovine achilles tendon collagen. *Biochemical and Biophysical Research Communications, 76*, 1124-1129.

Gallop, P.M., Blumenfeld, O. O., & Seifter, S. (1972). Structure and metabolism of connective tissue proteins. *Annual Reviews of Biochemistry, 41*, 617-672.

Garcia-Bunuel, L, & Garcia-Bunuel, V. M. (1967). Connective tissue and pentose phosphate pathway in normal and denervated muscle. *Nature, 4*, 913-914.

Garg, A. K., Berg, R. A., Silver, F. H., & Garg, H. G. (1989). Effect of proteoglycans on type I collagen fibre formation. *Biomaterials, 10 (6)*, 413-419.

Garret, R. R., & Flory, P. J. (1956). Evidence for a reversible first-order phase transition in collagen-diluent mixtures, *Nature, 177*, 176-177.

Gallop, P. M., Blumenfeld, O. O., & Seifter, S. (1972). Structure and metabolism of connective tissue proteins. *Annual Review of Biochemistry, 41*, 617-672.

Gatchalian, C. L., Schachner, M., & Sanes, J. R. (1989). Fibroblasts that proliferate near denervated synaptic sites in skeletal muscle synthesize the adhesive molecules tenascin (J1), N-CAM, fibronectin and a heparan sulphate proteoglycan. *The Journal of Cell Biology, 108,* 1873-1890.

Gault, N. F. S. (1985). The relationship between water-holding capacity and cooked meat tenderness in some beef muscles as influenced by acidic conditions below the ultimate pH. *Meat Science, 15*, 15-30.

Geesink, G. H., Koolmees, P. A., van Laack, H. L. J. M., & Smulders, F. J. M. (1995). Determinants of tenderization in beef longissimus dorsi and triceps brachi muscles. *Meat Science, 41* (1), 7-17.

Geesink, G. H., Kuchay, S., Chishti, A. H., & Koohmaraie, M. (2006). μ-Calpain is essential for postmortem proteolysis of muscle proteins. *Journal of Animal Science, 84*, 2834-2840.

Geeves, M. A., & Holmes, K. C. (1999). Structural mechanism of muscle contraction. *Annual Review Biochemistry, 68*, 687-728.

Góes, J. C., Figueiró, S. D., de Paiva, J. A. C., de Vasconcelos, I., F., & Sombra, A. S. B. (2002). On the piezoelectricity of anionic collagen films. *Journal of Physics and Chemistry of Solids, 63*, 465-470.

Goll, D.E., Bray, R.W., & Hoekstra, W.G. (1963). Age associated changes in muscle composition- Isolation and properties of collagenous residue from bovine muscle. *Journal of Food Science, 28*, 503-509.

Goll, D.E., Bray, R.W., & Hoekstra, W.G. (1964). Age –associated changes in bovine muscle connective tissue III. Rate of solubilization at 100 °C. *Journal of Food Science, 29*, 622-628.

Gómez-Guillén, M. C., Turney, J., Fernández-Díaz, M. D., Ulmo, N.,Lizarbe, M. A., & Montero, P. (2002). Structural and physical properties of gelatine extracted from different marine species: a comparative study. *Food Hydrocolloids, 16*, 25-34.

Gosline, J. M. (1978). Hydrophobic interaction and model for the elasticity of elastin. *Biopolymers, 17*, 677-695.

Gross, J., Highberger, J. H., & Schmitt, F. O. (1955). Extraction of collagen from connective tissue by neutral salt solutions. *Proceedings of the National Academy of Sciences, 41* (1), 1-7.

Gudiene, D., Valanciūte, A., & Velavicius, J. (2007). Collagen network changes in basilar artery in ageing. *Medicina, 43* (12), 964-970.

Haly, A. R., & Snaith, J. W. (1971). Calorimetry of rat tail tendon collagenbefore and after denaturation: the heat of fusion of its absorbed water. *Biopolymers, 10*, 1681-1699.

Hamling, A. E., & Calkins, C. R. (2008). Enhancement of beef chuck and loin muscles with ammonium hydroxide and salt. *Journal of Animal Science, 86*, 967-971.

Hamling, A. E., Jenschke, B. E., & Calkins, C. R. (2008). Effects of aging on beef chuck and loin muscles enhanced with ammonium hydroxide and salt. *Journal of Animal Science, 86,* 1200-1204.

Hampton, C. M., Taylor, D. W., & Taylor, K. A. (2007). Novel structure for α-actinin: F-actin interactions and their implications for actin-membrane attachment and tension sensing in the cytoskeleton. *Journal of Molecular Biology, 368*, 92-104.

Hanson, D. A., & Eyre, D. R. (1996). Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. *Journal of Biological Chemistry, 271* (43), 26508-26516.

Hardingham, T., & Bayliss, M. (1990). Proteoglycans of articular cartilage: changes in aging and joint disease. *Seminars in Arthritis and* Rheumatism, 20 (3), 12-33.

Harper, G. S. (1999). Trends in skeletal muscle biology and the understanding of toughness in beef. *Australian Journal of Agricultural Research, 50*, 1105-1129.

Harwalkar, V. R. & Ma, C. Y. (1987). Study of thermal properties of oat globulin by differential scanning calorimetry. *Journal of Food Science*, 52 (2), 394-398.

Hattori, A. and Takahashi, K. (1979). Studies on the post-mortem fragmentation of myofibrils. *Journal of Biochemistry, 85*, 47-56.

Hattori, S., Adachi, E., Ebihara, T., Shirai, T., Someki, I., & Irie, S. (1999). Alkali-treated collagen retained the triple helical conformation and ligand activity for the cell adhesion via α2β1 integrin. *Journal of Biochemistry, 125*, 676-684.

Hayashi, T., & Nagai, Y. (1980). The anomalous behaviour of collagen peptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis is due to the low content of hydrophobic amino acid residues. *Journal of Biochemistry, 87*, 803-808.

Hayashi, K., Markel, M. D., Thabit, G., Bogdanske, J. J., & Thielke, R. J. (1995). The effect of nonablative laser energy on joint capsular properties: an in vitro mechanical study using a rabbit model. *American Journal of Sports Medicine. 23*, 482-487.

Hayashi, K., Thabit, G., Massa, K. L., Bogdanske, J. J., Cooley, A. J., Orwin, J. F., & Markel, M. D. (1997). The effect of thermal heating on the length and histologic properties of the glenohumeral joint capsule. *The American Journal of the Sports Medicine, 25*, 107-112.

Hedrick, H. B., Stringer, W. C., Epley, R. J., Alexander, M. A., & Krause, G. F. (1968). Comparison of factors affecting Warner-Bratzler shear values of beef steaks. *Journal of Animal Science, 27*, 628-631.

Heinemeier, K. M., Olesen, J. L., Haddad, F., Langberg, H., Kjaer, M., Baldwin, K. M., & Schjerling, P. (2007). Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types. *Journal of Physiology, 582.3*, 1303-1316.

Henle, T. (2005). Protein bound advanced glycation end products (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids*, 29, 313-322.

Herbine, P., & Dyke, T. R., (1985). Rotational spectra and structure of the ammonia-water complex. *Journal of Chemical Physics, 83* (8), 3768-3774.

Herring, H. K. Cassens, R. G., & Briskey, E. J. (1965). Further studies on bovine muscle tenderness as influenced by carcass position, sarcomere length and fiber diameter. *Journal of Food Science, 30* (6), 1049-1054.

Herring, H. K. Cassens, R. G., & Briskey, E. J. (1967). Factors affecting collagen solubility in bovine muscles. *Journal of Food Science, 32*, 534-538.

Hill, F. (1966). The solubility of intramuscular collagen in meat animals of various ages. *Journal of Food Science*, *31*, 161-166.

Hodge, J. (1953). Dehydrated foods, chemistry of browning reactions in model systems, *Journal of Agriculture and Food Chemistry, 15*, 928-943.

Hoffman, L. C. (2006). Sensory and physical characteristics of enhanced vs. non-enhanced meat from mature cows. *Meat Science, 72*, 195-202.

Holmgren, S. K., Taylor, K. M., Bretscher, L. E., & Raines, R. T. (1998). Code for collagen's stability deciphered. *Nature, 392*, 666-667.

Honikel, K. O. (19980. Reference methods for the assessment of physical characteristics of meat. *Meat Science, 49* (4), 447-457.

Hopkins, D. L., & Thompson, J. M. (2001). The relationship between tenderness, proteolysis, muscle contraction and dissociation of actomyosin. *Meat Science, 57*, 1-12.

Hopkins, D. L., & Thompson, J. M. (2002). Factors contributing to proteolysis and disruption of myofibrillar proteins and the impact on tenderization in beef and sheep meat. *Australian Journal of Agricultural Research, 53*, 149-166.

Horgan, D. J., King, N. L., Kurth, L. B., & Kuypers, R. (1990). Collagen crosslinks and their relationship to the thermal properties of calf tendons. *Archives of Biochemistry and Biophysics, 281* (1), 21-26.

Horgan, D. J., Jones, P. N., King, Kurth, L. B., & Kuypers, R. (1991). The relationship between animal age and the thermal stability and cross-link content from five goat muscles. *Meat Science, 29*, 251-262.

Hörmann, H., & Schlebusch, H. (1971). Reversible and irreversible denaturation of collagen fibres. *Biochemistry, 10* (6), 932-937.

Höhne, G. W. H., Hemminger, W. F., & Flammersheim, H. J. *Differential Scanning Calorimetr*y, 2 nd ed.; Springer, New York, 2003; pp1-7.

Hostetler, R. L., Landmann, W. A., Link, B. A., & Fitzhugh, H. A. (1970). Influence of carcass position during rigor mortis on tenderness of beef muscles: Comparison of two treatments. *Journal of Animal Science, 31*, 47-50.

Huff, E. J., & Parrish, F. C. (1993). Bovine longissimus muscle tenderness as affected by postmortem aging time, animal age and sex. *Journal of Food Science, 58* (4), 713-716.

Huff-Lonergan, E., Parrish, F. C., & Robson, R. M. (1995). Effects of postmortem ageing time, animal age, and sex on degradation of titin and nebulin in bovine *longissimus* muscles. *Journal of Animal Science, 73*, 1064-1073.

Huffman, K. L., Miller, M. F., Hoover, L. C., Wu, C. K., Brittin, H. C., & Ramsey, C. B. (1996). Effect of beef tenderness on consumer satisfaction with steaks consumed in the home and restaurant. *Journal of Animal Science, 74*, 91-97.

Huijing, P. A. (1999). Muscular force transmission: a unified, dual or multiple system? A review and some explorative experimental results. *Archives of Physiology and Biochemistry, 107* (4), 292-311.

Hwang, I. H., Devine, C. E., & Hopkins, D. L. (2003). The biochemical and physical effects of electrical stimulation on beef and sheep meat tenderness. *Meat Science, 65*, 677-691.

Inerot, S., & Heinegård, D. (1978). Articular-cartilage proteoglycans in aging and osteoarthritis. *Biochemical Journal, 169,* 143-156.

Iqbal, M., Kenney, P. B., & Klandof, H. (1999). Age-related changes in meat tenderness and tissue pentosidines: Effect of diet restriction and aminoguanidine in broiler breeder hens. *Poultry Science, 78,* 1328-1333.

Jackson, M., Choo, L., Watson, P. H., Halliday, W. C., & Mantsch, H. H. (1995). Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues. *Biochemica et Biophysica Acta, 1270*, 1-6.

Jackson, M., Sowa, M. G., & Mantsch, H. H. (1997). Infrared spectroscopy: a new frontier in medicine. *Biophysical Chemistry, 68*, 109-125.

Janz, J. A. M., & Aalhus, J. A. The complexity of measuring beef tenderness using Warner-Bratzler shear methodology for whole muscle samples. In *Food Science and Product Technology*, T. Nakano & L. Ozimek, Eds.; Research Signpost, India. 2002, pp. 37-64..

Janolino, V. G., & Swaisgood, H. E. (1992). A spectrophotometric assay of solid phase primary amino groups. *Applied Biochemistry and Biotechnology, 36*, 81-85.

Jelesarov, I. & Bosshard, H. R. (1999). Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *Journal of Molecular Recognition*, 12, 3-18.

Jerez, N. C., Calkins, C. R., & Velazco, J. (2003). Prerigor injection using glycolytic inhibitors in low-quality beef muscles. *Journal of Animal Science, 81*, 997-1003.

Jordan, C. D., Charbonneau, N. L., & Sakai, L. Y. (2006). Fibrillin microfibrils: connective tissue pathways that regulate shape and signaling. *Journal of Musculoskeletal Neuronal Interact, 6* (4), 366-367.

Jurie, C., Picard, B., Hocquette, J. F., Dransfield, E., Micol, D., & Listrat, A. (2007). Muscle and meat quality characteristics of Holstein and Salers cull cows. *Meat Science, 77*, 459-466.

Kadler, K. E., Holmes, D.F., Trotter, J. A., & Chapman, J. A. (1996). Collagen fibril formation. *Biochemistry Journal, 316*, 1- 11.

Kagan, H. M., & Li, W. (2003). Lysyl oxidase: properties, specificity and biological roles inside and outside of the cell. *Journal of Cellular Biochemistry, 88*, 660-672.

Kakivaya, S. R., & Hoeve, C. A. J. (1975). The glass point of elastin. *Proceedings of National Academy of Sciences USA, 72* (9), 3505-3507.

Kamińska, A., & Sionkowska, A. (1996). Effect of UV radiation on the infrared spectra of collagen. *Polymer Degradation and Stability*, 51, 19-26.

Kanawa, R., Ji, J. R. & Takahashi, K. Inactivity of μ-calpain throught postmortem aging of meat. *Journal of Food Science, 67* (2), 635-638.

Kang, A. H., & Gross, J. (1970). Relationship between the intra and intermolecular cross-links of collagen. *Proceedings of the National Academy of Sciences, 67* (3), 1307-1314.

Kang, C. K., & Rice, E. F. (1970). Degradation of various meat fractions by tenderizing enzymes. *Journal of Food Science, 35*, 563-565.

Kang, C. K., & Warner, W. D. (1974). Tenderization of meat with papaya latex proteases. *Journal of Food Science, 39*, 812-818.

Kemp, P. D., & Scott, J. E. (1988). Ehrlich chromogens, probable cross-links in elastin and collagen. *Biochemical Journal, 252*, 387-393.

King, N. L. (1987). Thermal transition of collagen in ovine connective tissues. *Meat Science, 20*, 25-37.

Kirsch, K. M., Zelickson, B. D., Zachary, C. B., & Whitney, D. T. (1998). Ultrastructure of collagen thermally denatured by microsecond domain pulsed carbon dioxide laser. *Archives of Dermatology, 134,* 1255-1259.

Kim, C. W., Ho, G. P., & Ritchey, S. J. (1967). Collagen content and subjective scores for tenderness of connective tissue in animals of different ages. *Journal of Food Science*, *32*, 586-588.

Kisseleva, T., & Brenner, D. A. (2008). Mechanisms of fibrogenesis. *Experimental Biology and Medicine*, 233 (2), 109-122.

Kivirriko, K. I., & Myllyharju, J. (1998). Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biology, 16*, 357-368.

Knaggs, J. (1929). Studies on Collagen – The change which collagen undergoes when treated with solutions of hydrochloric acid and sodium hydroxide. *Biochemiocal Journal, 23* (6), 1308-1327.

Kohler, A., Bertrand, D., Martens, H., Hannesson, K., Kirschner, C., & Ofstad, R. (2007). Multivariate image analysis of a set of FTIR microspectroscopy images of aged bovine muscle tissue combining image and design information. *Analytical and Bioanalytical Chemistry, 389,* 1143-1153.

Kong, B., Diao, X., & Xiong, Y. L. (2006). Postmortem calcium chloride injection alters ultrastructure and improves tenderness of mature Chinese yellow cattle longissimus muscle. *Journal of Food Science, 71* (3), C124- C129.

Koniecko, E. S. *Hand Book of Meat Analysis*, 2nd ed.. Avery Publishing Group Inc., New Jersey, 1979.

Koohmaraie, M. & Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74*, 34-43.

Kovanen, V., Suominen, H., & Heikkinen, E. (1984). Collagen of slow twitch and fast twitch muscle fibres in different types of rat skeletal muscle. *European Journal of Applied Physiology, 52*, 235-242.

Kovanen, V. (2002). Intramuscular extracellular matrix: complex environment of muscle cells. *Exercise and Sport Sciences Reviews, 30* (1), 20-25.

Kragh, J. F., Svoboda, S. J., Wenke, J. C., Brooks, D. E., Bice, T. G., & Walters, T. J. (2005). The role of epimysium in suturing skeletal muscle lacerations. *Journal of American College of Surgeons, 200* (1), 38-44.

Kruggel, W. G., Field, R. A., & Miller, G. J. (1970). Physical and chemical properties of epimysial acid soluble collagen from meats of varying tenderness. *Journal of Food Science, 35*, 106-110.

Kulla, E., & Zuman, P. (2008). Reactions of orthophthalaldehyde with ammonia and 2-aminoethanol. *Organic and Biomolecular Chemistry, 6*, 3771-3780.

Kurose, T., Asai, Y., Mori, E., Daitoku, D., & Kawamata, S. (2006). Distribution and change of collagen types I, II and III and elastin in developing leg muscle in rat. *Hiroshima Journal of Medical Sciences, 55* (3), 85-91.

Kuypers, R., Tyler, M., Kurth, L. B., & Horgan, D. J. (1992). Identification of the loci of the collagen-associated Ehrlich chromogen in type I collagen confirms its role as a trivalent cross-link. *Biochemical Journal, 283*, 129-136.

Laakkonen, E., Wellington, G. H., & Sherbon, J. W. (1970). Low-temperature long time heating of bovine muscle: I. Changes in tenderness, water binding capacity, pH and amount of water-soluble components. *Journal of Food Science, 35*, 175-177.

Lahucky, R., Palanska, O., Mojto, J., Zaujec, K., & Huba, J. (1998). Effect of pre-slaughter handling on muscle glycogen level and selected meat quality traits in beef. *Meat Science, 50* (3), 389-393.

Lamare, M., Taylor, R. G., Farout, L., Briand, Y., & Briand, M. (2002). Changes in proteosome activity during postmortem aging of bovine muscle. *Meat Science, 61*, 199-204.

Laurent, G. J. (1987). Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. *American Journal of Physiology. Cell physiology, 252*, 1-9.

Lawrence, T. E., Dikeman, M. E., Stephens, J. W., Obuz, E., & Davis, J. R. (2003). In situ investigation of calcium-induced proteolytic and salting-in mechanisms causing tenderization in calcium-enhanced muscle. *Meat Science, 66*, 69-75.

Leander, R. C., Hedrick, H. B., Brown, M. F., & White, J. A. (1980). Comparison of structural changes in bovine *longissimus* and *semitendinosus* muscles during cooking. *Journal of Food Science, 45*, 1-6, 12.

LeBlanc, M. Chronology of BSE related events and government initiatives. Parliamentary Information and Research Service, Library of Parliament. http://www.parl.gc.ca/information/library/PRBpubs/prb0412-e.pdf , 2007.. (accessed on March 17, 2008).

Lehmann, K. B. (1907). Studien uber die zähigkeit des fleisches and ihre ursachen. *Archieves of Hygiene, 63*, 134-179. cited in Brady, D. E. (1937). A study of the factors influencing tenderness and texture of beef. *Journal of Animal Science, 1*, 246-250.

Lepetit, J. (2008). Collagen contribution to meat toughness: Theoretical aspects. *Meat Science*, 80, 960-967.

Lepetit, J. (2007). A theoretical approach of the relationships between collagen content, collagen cross-links and meat tenderness. *Meat Science, 76*, 147-159.

Lepetit, J., Grajales, A., & Favier, R. (2000). Modeling the effect of sarcomere length on collagen thermal shortening in cooked meat: consequences on meat toughness. *Meat Science, 54*, 239-250.

Lezina, V. P., Shirokova, L. V., Borunov, M. M., Stepanyants, A. U., & Smirnov, L. D. (1981). Temperature dependence of the ionization constants and pH values of aqueous solutions of 3-hydroxypyridine. *Russian chemical Bulletin, 30* (4), 540-544.

Li, C. B., Xu, X. L., Zhou, G. H., Xu, S. Q., & Zhang, J. B. (2007). Effects of carcass maturity on meat quality characteristics of beef *semitendinosus* muscle for chinese native yellow steers. *Animal, 1*, 780-786.

Lieber, R. L.. Skeletal muscle anatomy. In *Skeletal muscle structure and function: implications for rehabilitation and sports medicine*, Williams & Wilkins: Baltimore, 1992, pp. 1-45.

Light, N., & Champion, A. E. (1984). Characterization of muscle epimysium, perimysium and endomysium collagens. *Biochemical Journal, 219*, 1017-1026.

Light, N., Champion, A. E., Voyle, C., & Bailey, A. J. (1985). The role of epimysial, perimysial and endomysial collagen in determining texture in six bovine muscles. *Meat Science*, *13* (3), 137-149.

Lindahl, U., & Höök, M. (1978). Glycosaminoglycans and their binding to biological macromolecules. *Annual Review of Biochemistry, 47*, 385-417.

Listrat, A., Levieux, A., & Lepetit, J. (2007). Muscle, age and sex effects on total pyridinoline cross-links content and beef tenderness. *Proceedings of the 53rd international congress of meat science and technology*, Beijing, China, 247-248

Liu, A., Nishimura, T., & Takahashi, K. (1996). Relationship between structural properties of intramuscular connective tissue and toughness of various chicken skeletal muscles. *Meat Science, 43* (1), 43-49.

Lorenzen, C. L., Miller, R. K., Taylor, J. F., Neely, T. R., Tatum, J. D., Wise, J. W., Buyck, M. J., Reagan, J. O., & Savell, J. W. (2003). Beef customer satisfaction: trained sensory panel ratings and warner-bratzler shear force values. *Journal of Animal Science, 81*, 143-149.

Loucks, L. J., Ray, E. E., Berry, B. W., Leighton, E. A., & Gray, D. G. (1984). Effects of mechanical tenderization and cooking treatments upon product attributes of pre-and post-rigor beef roasts. *Journal of Animal Science, 58* (3), 626-630.

Lous, Le M., Flandin, F., Herbage, D., & Allain, J. (1982). Influence of collagen denaturation on the chemorheological properties of skin, assessed by differential scanning calorimetry and hydrothermal isometric tension measurement. *Biochimica et Biophysica Acta, 717* (2), 295-300.

Lovell, C. R., Smolenski, K. A., Duance, V. C, Light, N. D., Young, S., & Dyson, M. (1987). Type I and III collagen content and fibre distribution in normal human skin during ageing. *British Journal of Dermatology, 117* (4), 419-428.

Luescher, M., Rüegg , M. & Schindler. P. (1974). Effect of hydration upon the thermal stability of tropocollagen and its dependence on the presence of neutral salts. *Biopolymers, 13* (12) 2489-2503.

Lusk, J. L., Fox, J. A., Schroeder, T. C., Mintert, J., & Koohmararie, M. (2001). In-store evaluation of steak tenderness. *American Journal of Agricultural Economics, 83* (3), 539-550.

Luo, X., Zhu, Y., & Zhou, G. (2008). Electron microscopy of contractile bands in low voltage electrical stimulation beef. *Meat Science*, doi: 10.10.16/j.meatsci.2008.03.017.

Ma, H., Jiae, W., Huang, C., Wang, S., Chen, T., Cheng, C., & Hung, S. (2005). Thermal effects after anterior cruciate ligament shrinkage using radiofrequency technology: a porcine cadaver study. *Knee Surgery, Sports Traumatology, Arthroscopy, 13*, 619-624.

Mabrey, S. & Sturtevant, J. M. (1976). Investigation of phase transition of lipids and lipid mixtures by high sensitivity differential scanning calorimetry. *Proceedings of the National Academy of Sciences*, 73 (11), 3862-3866.

Maekawa, T., Rathinasamy, T. K., Altman, K. I., & Forbes, W. F. (1970). Changes in collagen with age-I. *Experimental Gerontology, 5,* 177-186.

Maher, S. C., Mullen, A. M., Buckley, D. J., Kerry, j. P., & moloney, A. P. (2005). The influence of biochemical differences on the variation in tenderness of *M.longissimus dorsi* of Belgian Blue steers managed homogeneously pre and post-slaughter. *Meat Science, 69*, 215-224.

Maher, S. C., Mullen, A. M., Moloney, A. P., Reville, W., Buckley, D. J., Kerry, J. P., & Troy, D. J. (2005). Ultrastructural variation in beef *M. longissimus dorsi* as an explanation of the variation in beef tenderness. *Journal of Food Science, 70* (9), E579-E584.

Maitland, J. D., Walsh, J. T. (1997). Quantitative measurements of linear birefringence during heating of native collagen. *Lasers in Surgery and Medicine, 20* (3), 310-318.

Maroudas, A., Evans, H., & Almeida, L. (1973). Cartilage of the hip joint. Topographical variation of glycosaminoglycan content in normal and fibrillated tissue. *Annals of the Rheumatic Diseases, 32*, 1-9.

Maroudas, A. (1976). Balance between swelling pressure and collagen tension in normal and degenarate cartilage. *Nature, 260*, 808-809.

Marsh, B. B. (1977). The basis of tenderness in muscle foods. *Journal of Food Science, 42* (2), 295-297.

Martens, H., Stabursvik, E., & Martens, M. (1982). Texture and colour changes in meat during cooking related to thermal denaturation of muscle proteins. *Journal of Texture Studies*, *13* (3), 291-309.

Martin, J. A., & Buckwalter, J. A. (2002). Aging, articular cartilage chondrocyte senescence and osteoarthritis. *Biogerontology, 3*, 257-264.

Martinez, G., & Millhauser, G. (1995). FTIR spectroscopy of alanine-based peptides: Assignment of the amide I mods for random coil and helix. *Journal of Structural Biology*, 114, 23-27.

Mays, P. K., McAnulty, R. J., Campa, J. S., & Laurent, G. F. (1991). Age-related changes in collagen synthesis and degradation in rat tissues. *Biochemical Journal, 276*, 307-313.

McClain, P. E. (1969). Isolation of intramuscular connective tissue. *Nature, 221*, 181-182.

McClain, P.E., & Wiley. E.R. (1972). Differential scanning calorimeter studies of the thermal transition of collagen. *The Journal of Biological Chemistry*. *247*(3): 692-697.

McCormick, R. J. (1994). The flexibility of collagen compartment of muscles. *Meat Science, 36*, 79-91.

McCormick, R. J. (1999). Extracellular modifications to muscle collagen: implications for meat quality. *Poultry Science, 78*, 785-791.

McIntosh, L. M., Jackson, M., Mantsch, H. H., Stranc, M. F., Pilavdzic, D. A., & Crowson, A. N. (1999). Infrared spectra of basal carcinomas are distinct from non-tumor-bearing skin components. *The Journal of Investigative Dermatology, 112* (6), 951-956.

McKeith, F. K., DeVol, D. L., Miles, R. S., Bechtel, P. J., & Carr, T. R. (1985). Chemical and sensory properties of thirteen major beef muscles. *Journal of Food Science, 50*, 869-872.

Mechanic, G. L., Katz, E. P., Henmi, M., Noyes, C., & Yamauchi, M. (1987). Locus of a histidine-based, stable trifunctional, helix to helix collagen cross-link: stereospecific collagen structure of type I skin fibrils. *Biochemistry, 26*, 3500-3509.

Meyer, M., & Morgenstern, B. (2003). Characterization of gelatin and acid soluble collagen by size exclusion chromatography coupled with multi angle light scattering (SEC-MALS). *Biomacromolecules. 4*, 1727-1732.

Meyer, K., & Rapport, M. M. (1951). The mucopolysaccharides of the ground substance of connective tissue. *Science, 113* (2943), 596-599.

Mikel, W. B., Goddard, B. L., & Bradford, D. D. (1996). Muscle microstructure and sensory attributes of organic acid-treated beef strip loins. *Journal of Food Science, 61* (5), 1058-1062.

Miles, C. A., Burjanadze, T. V., & Bailey, A. J. (1995). The kinetics of the thermal denaturation of collagen in unrestrained rat tail tendon determined by differential scanning calorimetry. *Journal of Molecular Biology, 245*, 437-446.

Miles, C. A., & Ghelashvili, M. (1999). Polymer-in-a-box mechanism for the thermal stabilization of collagen molecules in fibres. *Biophysical Journal, 76*, 3243-3252.

Miles, C. A, & Bailey, A. J. (2001). Thermally labile domains in the collagen molecule. *Micron*, 32, 325-332.

Miles, C. A., Avery, N. C., Rodin, V. V., & Bailey, A. J. (2005). The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. *Journal of Molecular Biology, 346*, 551-556.

Miller, M. F., Huffman, K. L., Gilbert, S. Y., Hamman, L. L., & Ramsey, C. B. (1995). Retail consumer acceptance of beef tenderized with calcium chloride. *Journal of Animal Science, 73*, 2308-2314.

Miller, M. F., Hoover, L. C., Cook, K. D., Guerra, A. L., Huffman, K. L., Tinney, K. S., Ramsey, C. B., Brittin, H. C., & Huffman, L. M. (1995). Consumer acceptability of beef steak tenderness in the home and restaurant. *Journal of Food Science, 60* (5), 963-965.

Miller, M. F., Carr, M. A., Ramsey, C. B., Crockett, K. L., & Hoover, L. C. (2001). Consumer thresholds for establishing the value of beef tenderness. *Journal of Animal Science, 79*, 3062-3068.

Minor, R. R. (1980). Collagen metabolism: a comparison of diseases of collagen and diseases affecting collagen. *American Journal of Pathology, 98* (1), 225-280.

Mirsky, A. E., & Pauling, L. (1936). On the structure of native, denatured and coagulated proteins. *Proceedings of the National Academy of Sciences of the United States of America, 22* (7), 439-447.

Mitchell, H. H., Hamilton, T. S., & Haines, W.T. (1928). Some factors affecting the connective tissue content of beef muscles. *Journal of Nutrition, 1* (2) 165-178.

Mitchell, H. H., Zimmerman, R. L., & Hamilton, T. S. (1927). The determination of the amount of connective tissue in meat. *The Journal of Biological Chemistry, 21* (2), 379-387.

Mizuta, S., Yamada, T., Miyagi, T., & Yoshinaka, R. (1999). Histological changes in collagen related to textural development of prawn meat during heat processing. *Journal of Food Science, 64* (6), 991-995.

Møller**,** A.J. (1981). Analysis of Warner-Bratzler shear pattern with regard to myofibrillar and connective tissue components of tenderness. *Meat Science*, *5,* 247-260.

Möller**,** C., Steinhart, H., & Bosselmann, A. (1993). Isolation of bovine intramuscular connective tissue. *Z Lebensum Unters Forsch, 196*, 415-416.

Molnar, J. A., Nathaniel, A., Burke, J. F., & Young, V. R. (1986). Synthesis and degradation rates of collagen in vivo in whole skin of rats, studied with $^{18}O_2$ labeling. *Biochemical Journal, 240*, 431-435.

Monnier, V. M., Mustata, G. T., Biemel, K. L., Reihl, O., Lederer, M. O., Zhenyu, D., & Sell, D. R. (2005). Cross-linking of the extracellular matrix by the Maillard reaction in aging and diabetes. A*nnals of the New York Academy of Sciences, 1043*, 533-544.

Monsón, F., Sañudo, C., & Sierra, I. (2004). Influence of cattle breed and ageing time on textural meat quality , *Meat Science, 68*, 595-602.

Morgan, J. B., Savell, J. W., Hale, D. S., Miller, R. K., Griffin, D. B., Cross, H. R., & Shakelford, S. D. (1991). National beef tenderness survey. *Journal of Animal Science, 69*, 3274-3283.

Moriguchi, T., & Fujimoto, D. (1978). Age-related changes in the content of the collagen crosslink, pyridinoline. *Journal of Biochemistry, 84*, 933-935.

Myllyharju, J. (2003). Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biology.* 22, 15-24.

Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material- skin, bone and fins. *Food Chemistry, 68*, 277-281.

Nagainis, P., & Wolfe, F. H. (1982). Calcium activated neutral protease hydrolyzes Z-disc actin. *Journal of Food Science, 47*, 1358-1364.

Nagaraj, N. S., Anilkumar, K. R., & Santhanam, K. (2005). Postmortem changes in myofibrillar proteins of goat skeletal muscles. *Journal of Food Biochemistry, 29*, 152-170.

Nakamura, Y., Iwamoto, H., Yamaguchi, T., Ono, Y. Nakanishi, Y., Tabata, S., Nishimura, S., & Gotoh, T. (2007). Three-dimensional reconstruction of intramuscular collagen networks of bovine muscle: a demonstration by an immunohistochemical/confocal laser-scanningmicroscopic method. *Animal Science Journal, 78*, 445-447.

Nakano, T., Sunwoo, H. H., Li, X., Price, M. A., & Sim, J. S. (1996). Study of sulfated glycosaminoglycans from porcine skeletal muscle epimysium including analysis of iduronosyl and glucuronosyl residues in galactoseaminoglycan fractions. *Journal of Agriculture and Food Chemistry, 44* (6), 1424-1434.

Neely, T. R., Lorenzen, C. L., Miller, R. K., Tatum, J. D., Wise, J. W., Taylor, J. F., Buyck, M. J., Reagan, J. O., & Savell, J. W. (1998). Beef customer satisfaction: role of cut, USDA quality grade, and city on in-home consumer ratings. *Journal of Animal Science, 76*, 1027-1033.

Nelson, Jr. D. D., Fraser, G. T., & Klemperer, W. (1987). Does ammonia hydrogen bond? *Science, 238,* 1671-1674.

Nēmethy, G., & Scheraga, H. A. (1986). Stabilization of collagen fibrils by hydroxyproline. *Biochemistry, 25*, 3184-3188.

Neuman, R. E., & Logan, M. (1950). The identification of collagen and elastin in tissues. *The Journal of Biological Chemistry, 186* (2), 549-556.

Newbold, R. P., & Harris, P. V. (1972). The effect of pre-rigor changes on meat tenderness. *Journal of Food Science, 37*, 337-340.

Nishimura, T., Hattori, A., & Tahahashi, K. (1994). Ultrastructure of the intramuscular connective-tissue in bovine skeletal muscle. *Acta Anatomica, 151* (4), 250-257.

Nishimura, T., Hattori, A., & Takahashi, K. (1995). Structural weakening of intramuscular connective tissue during conditioning beef. *Meat Science, 39,* 127-133.

Nishimura, T., Ojima, K., Hattori, A., & Takahashi, K. (1997). Developmental expression of extracellular matrix components in intramuscular connective tissue of bovine semitendinosus muscle. *Histochemistry and Cell biology, 107* (3), 215-221.

Nishimura, T., Hattori, A., & Takahashi, K. (1999). Structural changes in intramuscular connective tissue during the fattening of Japanese black cattle: effect of marbling on beef tenderization. *Journal of Animal Science, 77*, 93-104.

Nishimura, T., Taneichi, A., Wakamatsu, J., & Hattor, A. (2003). Effect of skeletal muscle decorin on collagen fibrillagenosis in vitro. *Animal Science Journal, 74* (5), 399-405.

Norman, G. A. (1982). Effect of breed and nutrition on the productive traits of beef cattle in South-East Brazil: Part 3-Meat Quality. *Meat Science, 6,* 79-96.

Öbrink, B., & Sundelöf, L. (1973). Light scattering in the study of associating macromolecules. *European Journal of Biochemistry, 37*, 226-232.

Obuz, E., Dikeman, M. E., & Loughin, T. M. (2003). Effects of cooking method reheating holding time and holding temperature on beef longissimus lumborum and biceps femoris tenderness. *Meat Science, 65*, 841-851.

Okamoto, Y., & Saeki, K. (1964). Phase transition of collagen and gelatin. *Colloid & Polymer Science*, 194 (2), 124-135.

Okamoto, Y., Heeley, J. D., Dogon, I. L., & Shintani, H. (1991). Effects of phosphoric acid and tannic acid on dentine collagen. *Journal of Oral Rehabilitation, 18*, 507-512.

Oleggini, R., Gastaldo, N., & Di Donato, A. (2007). Regulation of elastin promoter by lysyl oxidase and growth factors: cross control of lysyl oxidase on TGF-β 1 effects. *Matrix Biology, 26,* 494-505.

Olkowski, A. A., Rathgeber, B. M., Sawicki, G., & Classen, H. L. (2001). Ultrastructural and molecular changes in the left and right ventricular myocardium associated with ascites syndrome in broiler chickens raised at low altitude. *Journal of Veterinary Medicine A, 48*, 1-14.

Olson, D.G., Parrish, F. C., & Stromer, M. H. (1976). Myofibril fragmentation and shear resistance of three bovine muscles during postmortem storage. *Journal of Food Science, 41*, 1036-1041.

Oreskovich, D. C., Bechtel, P. J., McKeith, F. K., Novakofski, J., & Basgall, E. J. (1992). Marinade pH affects textural properties of beef. *Journal of Food Science, 57* (2), 305-311.

Ouali, A. (1990). Meat tenderization: Possible causes and mechanisms. A review. *Journal of Muscle Foods, 1*, 129-165.

Overbeek, J., & Th, G.(1956). The donnan equilibrium. *Progress in Biophysics & Biophysical Chemistry, 6*, 57-84.

Oxlund, H., Barckman, M., Ørtoft, G., & Andreassen, T. T. (1995). Reduced concentrations of collagen cross-links are associated with reduced strength of bone. *Bone, 17* (4), 365S-371S.

Oxlund, H., Sekilde, L., & Ørtoft, G. (1996). Reduced concentration of collagen reducible cross links in humantrabecular bone with respect to age and osteoporosis, *Bone, 19* (5), 479-484.

Palka, K. (1999). Changes in intramuscular connective tissue and collagen solubility of bovine *m. semitendinosus* during retorting. *Meat Science, 53*, 189-194.

Palokangas, H., Kovanen, V., Duncan, A., & Robins, S. P. (1992). Age-related changes in the concentration of hydroxypyridinium crosslinks in functionally different skeletal muscles. *Matrix, 12* (4), 291-296.

Panick, G., Malessa, R., & Winter, R. (1999). Differences between the pressure and temperature induced denaturation and aggregation of β-lactoglobulin A, B, and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry, 38*, 6512-6519.

Park, B., Whittaker, A. D., Miller, R. K., & Hale, D. S. (1994). Ultrasonic spectral analysis for beef sensory attributes. *Journal of Food Science, 59* (4), 697-701.

Parrish, F. C., Vandell, C. J., & Culler, R. D. (1979). Effect of maturity and marbling on the myofibril fragmentation index of bovine *longissimus* muscle. *Journal of Food Science, 44*, 1668-1671.

Paschalis, E. P., Verdelis, K., Doty, S. B., Boskey, A. L., Mendelsohn, R., & Yamauchi, M. (2001). Spectroscopic characterization of collagen cross-links in bone. *Journal of Bone and Mineral Research*, 16(10), 1821-1828.

Passerieux, E., Rossignol, R., Chopard, A., Carnino, A., Marini, J. F., Letellier, T., & Delage, J. P. (2006). Structural organization of the perimysium innbovine skeletal muscle: junctional plates and associated intramuscular subdomains. *Journal of Structural Biology, 154*, 206-216.

Passerieux, E., Rossignol, R., Letellier, T., & Delage, J. P. (2007). Physical continuity of the perimysium from myofibres to tendons: Involvement in lateral force transmission in skeletal muscle. *Journal of Structural Biology, 159*, 19-28.

Peachey, B. M., Purchas, R. W., & Duizer, L. M. (2002). Relationships between sensory and objective measures of meat tenderness of beef m. longissimus thoracis from bulls and steers. *Meat Science, 60*, 211-218.

Pearson, A. M., & Young, R. B. *Muscle and meat biochemistry*. Academic Press: California, 1989, pp. 338-390.

Pelpis, A. M. D. G, Goissis, G., & Das-Gupta, D. K. (1996). Dielectric and pyroelectric characterization of anionic and native collagen. *Polymer Engineering and Science, 36* (24), 2932-2938.

Pérez-Tamayo, R. (1978). Pathology of collagen degradation. *American Journal of Pathology, 92* (2), 508-566.

Perkins, M. E., Ji, T. H., & Hynes, R. O. (1979). Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. *Cell, 16*, 941-952.

Pfeiffer, K. D., Voges, K. L., King, D. A., Griffin, D. B., & Savell, J. W. (2005). Innovative wholesale carcass fabrication and retail cutting to optimize beef value. *Meat Science, 71*, 743-752.

Pierschbacher, M. D., & Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature, 309*, 30-33.

Pietrasik, Z., & Shand, P. J. (2004). Effect of blade tenderization and tumbling time on the processing characteristics and tenderness of injected cooked roast beef. *Meat Science, 66*, 871-879.

Piez, K. A., Martin, G. R., Kang, A. H., & Bornstein, P. (1966). Heterogeneity of the α chains of rat skin collagen and its relation to the biosynthesis of cross-links. *Biochemistry, 5* (12), 3813-3820.

Plepis, A. M. D. G., Goissis, G., & Das-Gupta, D. K. (1996). Dielectric and pyroelectric characterization of anionic and native collagen. Polymer Engineering and Science, 36 (24), 2932-2938.

Pokharna, H. K., & Pottenger, L.A. (1997). Nonenzymtic glycation of cartilage proteoglycans: an in vivo and in vitro study. *Glycoconjugate Journal, 14*, 917-923.

Pommier, S. A. (1992). Vitamin A, electric stimulation, and chilling rate effects on lysosomal enzyme activity in aging bovine muscles. *Journal of Food Science, 57* (1), 30-35.

Potter, K., Kidder, L. H., Levin, I. W., & Lewis, E. N. (2001). Imaging of collagen and proteoglycan in cartilage sections using fourier transform infrared spectral imaging. *Arthritis & Rheumatism, 44* (4), 846-855.

Poulin, D., & Boame, A. K.. *Mad cow disease and beef trade.* Statistics Canada. http://www.statcan.gc.ca/english/research/11-621-MIE/11-621-MIE2003005.pdf. 2003. (accessed March 14, 2008).

Powell, T. H., Hunt, M. C., & Dikeman, M. E. (2000). Enzymatic assay to determine collagen thermal denaturation and solubilization. *Meat Science, 54*, 307-311.

Prost, E., Pełczyńska, E., & Kotula, A. W. (1975). Quality characteristics of bovine meat. 1. Content of connective tissue in relation to individual muscle, age and sex of animals and carcass quality grade. *Journal of Animal Science, 41* (2), 534-540.

Prost, E., Pelczynska, E., & Kotula, A. W. (1975). Quality characteristics of bovine meat. II. Beef tenderness in relation to individual muscles, age and sex of animals and carcass quality grade. *Journal of Animal Science, 41*, 541-547.

Purslow, P.P. (1999). Intramuscular connective tissue and its role in meat quality. *Meat Science, 70*, 435-447.

Purslow, P. P. (1989). Strain-induced reorientation of an intramuscular connective tissue network: implications for passive muscle elasticity. *Journal of Biomechanics, 22* (1), 21-31.

Purslow, P. P. (2005). Intramuscular connective tissue and its role in meat quality. *Meat Science, 70*, 435-447.

Quarrier, E., Carpenter, Z. L., & Smith, G. C. (1972). A physical method to increase tenderness in lamb carcasses. *Journal of Food Science, 37*, 130-131.

Quinn, J. R., Raymond, D. P. & Harwalkar, V. R. (1980). Differential scanning calorimetry of meat proteins as affected by processing treatment. *Journal of Food Science*, 45 (5), 1146-1149.

Radhika, M., & Sehgal, P. K. (1996). Studies on desamidation of bovine collagen. *Journal of Biomedical Materials Research, 35* (4), 497-503.

Ramachandran, G. N. & Kartha, G. (1954). Structure of Collagen. *Nature, 174, 269-270.*

Ramachandran, G. N. & Kartha, G. (1955). Structure of Collagen. *Nature, 176, 593-595.*

Ramachandran, G. N. (1988). Stereochemistry of collagen. *International Journal of Peptide and Protein Research*, *31* (1), 1-16.

Ramirez, F., Sakai, L. Y., Rifkin, D. B., & Dietz, H. C. (2007). Extracellular microfibrills in development and disease. *Cellular and Molecular Life Sciences, 64*, 2437-2446.

Reagan, J. O., Carpenter, Z. L., & Smith, G. C. (1976). Age-related traits affecting the tenderness of the bovine longissimus muscle. *Journal of Animal Science, 43*, 1198-1205.

Reddy, G. K. (2004a), Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit Achilles tendon. *Experimental Diabesity Research*, 5, 143-153.

Reddy, G. K. (2004b). AGE-related cross-linking of collagen is associated with aortic wall matrix stiffness in the pathogenesis of drug-induced diabetes in rats. *Microvascular Research, 68*, 132-142.

Renugopalakrishnan, V., Chandrakasan, G., Moore, S., Hutson, T. B., Berney, C. V., & Bhatnagar, R. S. (1989). Bound water in collagen. Evidence from fourier transform infrared and fourier transform infrared photoacoustics spectroscopic study. *Macromolecules, 22* (10), 4121-4124.

Reuter, B.J., Wulf, D. M., & Maddock, R. J. (2002). Mapping intramuscular tenderness variation in four major muscles of the beef round. *Journal of Animal Science*, *80*, 2594-2599.

Rhee, M.S., Wheeler, T. L., Shackelford, S. D., & Koohmaraie, M. (2004). Variation in palatability and biochemical traits within and among eleven beef muscles. *Journal of Animal Science*, *82* (2), 534-550.

Rho, J., Kuhn-Spearing, L., & Zioupos, P. (1998). Mechanical properties and hierarchical structure of bone. *Medical Engineering and Physics, 20*, 92-102.

Rhodes, D. N., Jones, R. C. D., Chrystall, B. B., & Harries, J. M. (1972). Meat texture: II. The relationship between subjective assessment and compressive test on roast beef. *Journal of Texture Studies, 3* (3), 298-309.

Rhodes, R. K., & Miller, E. J. (1978). Physicochemical characterization and molecular organization of the collagen A and B chains. *Biochemistry, 17* (17), 3442-3448.

Rich, A. & Crick, F. H. C. (1995). The structure of collagen. *Nature, 176*, *915-916.*

Riley, D. G., Johnson, D. D., Chase, C. C., West, R. L., Coleman, S. W., Olsen, T. A., & Hammond, A. C. (2005). Factors influencing tenderness in steaks from Brahman cattle. *Meat Science, 70*, 347-356.

Robert, L., Jacob, M. P., Frances, C., Godeau, G., & Hornebeck, W. (1984). Interaction between elastin and elastases and its role in the aging of the arterial wall, skin and other connective tissues. *Mechanisms of Ageing and Development, 2 & 3*, 155-156.

Robert, L., Robert, A. M., & Fülöp, T. (2008). Rapid increase in human life expectancy: will it soon be limited by the aging of elastin? *Biogerontology, 9* (2), 119-133.

Robertson, J., Ratcliff, D., Bouton, P. E., Harris, P. V., & Shorthose, W. R. (1984). Effect of cooking temperature and animal age on the shear properties of beef and buffalo meat. *Journal of Food Scienec, 49*, 1163-1166.

Robins, S. P. (2007). Biochemistry and functional significance of collagen cross-linking. *Biochemical Society Transactions, 35* (5), 849-852.

Robins, S. P., Shimokomaki, M., & Bailey, A. J. (1973). The chemistry of collagen cross-links. Age related changes in the reducible components of intact bovine collagen fibres. *Biochemical Journal, 131*, 771-780.

Rochdi, A., Foucat, L., & Renou, J. P. (1999). Effect of thermal denaturation on water-collagen interactions: NMR relaxation and differential scanning calorimetry analysis. *Biopolymers, 50*, 690-696.

Rochdi, A., Foucat, L., & Renou, J. P. (2000). NMR and DSC studies during thermal denaturation of collagen. *Food Chemistry, 69*, 295-299.

Rodgers, U. R., & Weiss, A. S. (2005). Cellular interactions with elastin. *Pathologie Biologie, 53*, 390-398.

Rolan, T. L., Davis, G. W., Seidman, S. C., Wheeler, T. L., & Miller, M. F. (1988). Effects of blade tenderization and proteolytic enzymes on restructured steaks from beef bullock chucks. *Journal of Food Science, 53* (4). 1062-1064.

Romans, J. R., Tuma, H. J., & Tucker, W. L. (1965). Influence of carcass maturity and marbling on the physical and chemical characteristics of beef. I. Palatability, fibre diameter and proximate analysis. *Journal of Animal Science, 24*, 681-685.

Rooyackers, O. E., & Nair, S. (1997). Hormonal regulation of human muscle protein metabolism. *Annual Reviews Nutrition. 17*, 457-485.

Rosenvold, K., North, M., Devine, C., Micklander, E., Hansen, P., Dobbie, P., & Wells, R. (2008). The protective effect of electrical stimulation an wrapping on beef tenderness at high pre rigor temperatures. *Meat Science, 79*, 299-306.

Rosmus, L., Deyl, Z., & Drake, M. P. (1967). Studies on the structure of collagen, I the sequence analysis of peptides released by pronase. *Biochemica et Biophysica Acta, 140*, 507-514.

Rowe, R. W. D. (1981). Morphology of perimysial and endomysial connective tissue in skeletal muscle. *Tissue & Cell, 13* (4), 681-690.

Rowe, R. W. D. (1974). Collagen fibre arrangement in intramuscular connective tissue. Changes associated with muscle shortening and their possible relevance to raw meat toughness measurement. *Journal of Food Technology, 9*, 501-508.

Rowe, R. W. D. (1986). Elastin in bovine semitendinosus and longissimus dorsi muscles. *Meat Science*, *17*, 293-312.

Rude, J., Carlberg, J., & Pellow, S. (2007). Integration to fragmentation: Post-BSE Canadian cattle markets, processing capacity and cattle prices. *Canadian Journal of Agriculture Economics, 55*, 197-216.

Ruoslahti, E. (1988). Structure and biology of proteoglycans. *Annual Reviews of Cell Biology, 4*, 229-255.

Sakai, L. Y., Keene, D. R., & Engvall, E. (1986). Fibrillin, a new 350kD glycoprotein, is a component of extracellular microfibrils. *The Journal of Cell Biology, 103* (6), 2499-2509.

Samejima, K., Ishioroshi, M., & Yasui, T. (1983). Scanning calorimetric studies on thermal denaturation of myosin and its subfragments. *Agricultural and Biological Chemistry, 47* (10), 2373-2380.

Sanui, K., MacKnight, W. J., & Lenz, R. W. (1974). The far-infrared spectrum of collagen. *Macromolecules, 7* (6), 954-956.

Schmidt, J. G., & Parrish, F. C. (1971). Molecular properties of postmortem muscle. 10. Effect of internal temperature and carcass maturity on structure of bovine longissimus. *Journal of Food Science, 36*, 110-119.

Schwartz, W. N., & Bird, J. W. C. (1977). Degradation of myofibrillar proteins by cathepsins B and D. *Biochemical Journal, 167*, 811-820.

Scott, J. E., Hughes, E. W., & Shuttleworth, A. (1981). A collagen-associated Ehrlich chromogen: a pyrrolic cross-link? *Bioscience Reports, 1*, 611-618.

Scott, J. E. (1988). Proteoglycan-fibrillar collagen interactions. *Biochemical Journal, 252*, 313-323.

Scott, J. E., Qian, R., Henkel, W., & Glanville, R. W. (1983). An Ehrlich chromogen in collagen cross-links. *Biochemical Journal, 209*, 263-264.

Scott, J. E. (1988). Proteoglycan-fibrillar collagen interactions. *Biochemical Journal, 252*, 313-323.

Scott, J. E., Bosworth, T. R., Cribb, A. M., & Taylor, J. R. (1994) The chemical morphology of age-related changes in human intervertebral disc glycosaminoglycans from cervical, thoracic and lumber nucleus pulposus and annulus fibrosus. *Journal of Anatomy, 184*, 73-82.

Seideman, S. C., & Cross, H. R. (1982). The economics and palatability attributes of hot-boned beef: A review. *Journal of Food Quality, 5* (3), 183-201.

Seideman, S. C. (1986). Methods of expressing collagen characteristics and their relationship to meat tenderness and muscle fibre types. *Journal of Food Science, 51* (2), 273-276.

Seideman, S. C., Cross, H. R., & Crouse, J. D. (1986). Use of mechanical tenderization to increase the tenderness of bullock beef. *Journal of Food Quality, 9*, 49-56.

Seideman, S. C., Smith, G. C., Carpenter, Z. L., & Marshall, W. H. (1977). Blade tenderization of beef psoas major and semitendinosus muscles. *Journal of Food Science, 42* (6). 1510-1512.

Seideman, S. C., & Theer, L. K. (1986). Relationships of instrumental textural properties and muscle fibre types to the sensory properties of beef. *Journal of Food Quality, 9*, 251-261.

Shackelford, S. D., Morgan, J. B., Cross, H. R., & Savell, J. W. (1991). Identification of threshold levels for Warner_Bratzler shear force in beef top loin steaks. *Journal of Muscle Foods, 2,* 289-296.

Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (1995). Relationship between shear force and trained sensory panel tenderness ratings of 10 major muscles from *Bos indicus* and *Bos taurus* cattle. *Journal of Animal Science, 73*, 3333-3340.

Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (1997). Repeatability of tenderness measurements in beef round muscles. *Journal of Animal Science, 75*, 2411-2416.

Shellswell, G. B., Bailey, A. J., Duance, V. C., & Restall, D. J. (1980). Has collagen a role in muscle pattern formation in the developing chick wing? 1. An immunofluorescence study. *Journal of Embryology and Experimental Morphology, 60*, 245-254.

Shimada, K., & Takahashi, K. (2003). Relationship between fragmentation of myofibrils and liberation of phospholipids from Z-disks induced by calcium ions at 0.1 mM: Mechanism of tenderization of pork and beef during postmortem ageing. *Journal of Food Science, 68* (9), 2623-2629.

Shimokomaki, M., Elsden, D. F., & Bailey, A. J. (1972) Meat tenderness: age related changes in bovine intramuscular collagen. *Journal of Food Science, 37*, 892-896.

Shorthose, W. R., & Harris, P. V. (1990). Effect of animal age on the tenderness of selected beef muscles. *Journal of Food Science, 55* (1), 1-8 &14.

Sifre, L., Berge, P., Engel, E., Martin, J., Bonny, J., Listrat, A., Taylor, R., & Culioli, J. (2005). Influence of the spatial organization of the perimysium on beef tenderness. *Journal of Agricultural Food Chemistry, 53*, 8390-8399.

Silva, J. A., Patarata, L., & Martins, C. (1999). Influence of ultimate pH on bovine meat tenderness during ageing. *Meat Science, 52*, 453-459.

Singh, R., Barden, A., Mori, T., & Beilin, L. (2001). Advanced glycation end products: a review. *Diabetologia, 44*, 129-146.

Sini, P., Denti, A., Tira, M. E., & Balduini, C. (1997). Role of decorin on in vitro fibrillogenesis of type I collagen. *Glycoconjugate Journal, 14*, 871-874.

Sivertsen, H. K., Kubberød, E., & Hildrum, K. I. (2002). Consumer preferences of beef tenderness and mechanical measurements. *Journal of Sensory Studies, 17*, 365-378.

Smith, C. K., Withka, J. M., & Regan, L. (1994). A thermodynamic scale for the β-sheet forming tendancies of the amino acids. *Biochemistry, 33*, 5510-5517.

Smith, G. C., Carpenter, Z. L., & King, G. T. (1969). Considerations for beef tenderness evaluations. *Journal of Food Science, 34*, 612-618.

Smith, K., & Rennie, M. J. (2007). New approaches and recent results concerning human-tissue collagen synthesis. *Current Opinion in Clinical Nutrition and Metabolic Care, 10* (5), 582-590.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*, 76-85.

Smith, S. H., & Judge, M. D. (1991). Relationship between pyridinoline concentration and thermal stability of bovine intramuscular collagen. *Journal of Animal Science, 69,* 1989-1993.

Smulders, F. J. M., Marsh, B. B., Swartz, D. R., Russel, R. L., & Hoenecke, M. E. (1990). Beef tenderness and sarcomere length, *Meat Science, 28*, 349-363.

Snowden, J. McK., Bouton, P. E., & Harris, P. V. (1977). Influence of constraint during heating and cooling on the mechanical properties of collagenous tissue. *Journal of Food Science, 42* (4).890-894.

Snowden, J. McK., & Weidemann, J. F. (1978). A morphological and biochemical examination of the hydrothermal denaturation of collagen. *Meat Science, 2* (1), 1-18.

Stanley, D. W., & Swatland, H. J. (1976). The microstructure of muscle tissue- a basis for meat texture measurement. *Journal of Texture Studies, 7* (1), 65-75.

Stathakis, N. E., & Mosesson, M. W. (1977). Interaction among heparin, cold-insoluble globulin and fibrinogen in formation of the heparin-precipitable fraction of plasma. *The Journal of Clinical Investigation, 60*, 855-865.

Stell, G., & Joslin, C. G. (1986). The donnan equilibrium- A theoretical study of the effects of interionic forces. *Biophysics Journal, 50*, 855-859.

Strydom, P. E., Naude, R. T., Smith, M. F., Scholtz, M. M., & van Wyk, 2000. Characterization of indigenous African cattle breeds in relation to meat quality traits. *Meat science, 55*, 79-88.

Sue, K., Usami, T., & Arai, K. (2003). Determination of acetic acid dissociation constants to 400 ºC and 32 Mpa by potentiometric pH measurements. *Journal of Chemical and Engineering Data, 48*, 1081-1084.

Surewicz, W. K., Mantsch, H. H., & Chapman, D. (1993). Determination of protein secondary structure by fourier transform infrared spectroscopy: A critical assessment. *Biochemistry, 32* (2), 389-394.

Sugawara, K., & Oyama, F. (1981). Fluorogenic reaction and specific microdetermination of ammonia. *Journal of Biochemistry, 89*, 771-774.

Swatland, H. J. The commercial structure of the carcass. In *Structure and Development of Meat Animals and Poultry*. Technomic Publishing Co.; Pennsylvania, 1994, pp. 143-196.

Swatland, H. J., & Findlay, C. J. (1997). On-line probe prediction of beef toughness, correlating sensory evaluation with fluorescence detection of connective tissue and dynamic analysis of overall toughness. *Food Quality and Preference, 8* (3), 233-239.

Swatland, H. J. (2006). Stratification of connective tissue toughness in beef roasts assessed by simultaneous fluorometry and penetrometry. *Food Research International, 39*, 1106-1109.

Swatland, H. J., (2007). Stratification of toughness in beef roasts. *Meat Science, 77*, 2-6.

Sparling, D. H., & Caswell, J. A. (2006). Risking market integration without regulatory integration: The case of NAFTA and BSE. *Review of Agricultural Economics, 28* (2), 212-228.

Stuart, A. E., Sofos, J. N., & Schmidt, G. R. (1991). Differential scanning calorimetric studies of meat protein-alginate mixtures. *Journal of Food Science, 56* (1), 175-179.

Tabata, S., Morita, S., Watanabe, T., Goto, T., Nishimura, S., Koga, Y., & Iwamoto, H. (1999). The collagen architecture and immunohistochemical property in bovine biceps femoris muscle. *Shokuniku ni kansuru Josei Kenkyu Chosa Seika Hokokusho, 17*, 171-174.

Takahashi, K., Shirai, K., & Wada, K. (1988). Melting behaviour of gels prepared from isolated subunits of collagen. *Journal of Food Science, 53* (6), 1920-1921.

Tan, J. (2004). Meat quality evaluation by computer vision. *Journal of Food Engineering, 61*, 27-35.

Tappel, A. L., Miyada, D. S., & Sterling, C. (1956). Meat tenderization. II. Factors affecting the tenderization of beef by papain. *Journal of Food Science, 21* (3), 375-383.

Tatum, J. D., Smith, G. C., & Carpenter, Z. L. (1978). Blade tenderization of steer, cow and bull beef. *Journal of Food Science, 43* (3), 2652-2660.

Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M., & Goll, D. E. (1995). Is Z-disk degradation responsible for postmortem tenderization? *Journal of Animal Science, 73*, 1351-1367.

Theis, E. R., & Jacoby, T. F. (1943). The acid and base binding capacity of heat denatured collagen. *The Journal of Biological Chemistry, 148*, 105-110.

Thieszen, S. L., & Rosenquist, T. H. (1994). Expression of collagens and decorin during aortic arch artery development: implications for matrix patter formation. *Matrix Biology, 14*, 573-582.

Thomas, L. C. Why modulated DSC®?; *An overview and summary of advantages and disadvantages relative to traditional DSC*. TA Instruments, New Castle, 2005, Pp.1-8.

Thompson, E. H., Wolf, I. D., & Allen, C. E. (1973). Ginger rhizome: A new source of proteolytic enzyme. *Journal of Food Science, 38,* 652-655.

Torikai, A., & Shibata, H. (1999). Effect of ultraviolet radiation on photodegradation of collagen. *Journal of Applied Polymer Science, 73*, 1259-1265.

Tornberg, E. (1996). Biophysical aspects of meat tenderness. *Meat Science, 43* (S), 175-191.

Torrescano, G., Sánchez-Escalante, A., Giménez, B., Roncalés, P., & Beltrán, J. A. (2003). Shear values of raw samples of 14 bovine muscles and their relation to muscle collagen characteristics. *Meat Science, 64,* (1) 85-91.

Torzilli, P. A., Grigiene, R., Borrelli, J., & Helfet, D. L. (1999). Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. *Journal of Biomechanical Engineering, 121*, 433-441.

Trop, M., & Birk, Y. (1970). The specificity of proteinases from Streptomyces griseus (pronase). *Biochemical Journal, 116*, 19-25.

Trotter, J. A., & Purslow, P. P. (1992). Functional morphology of the endomysium in series fibered muscles. *Journal of Morphology, 212* (2), 109-122.

Tsugita, A., & Schefler, J. (1982). A rapid method for acid hydrolysis of protein with a mixture of trifluoroacetic acid and hydrochloric acid. *European Journal of Biochemistry, 124,* 585-588.

Uitto, J. (1979). Biochemistry of elastic fibres in normal connective tissues and its alterations in diseases. *The Journal of Investigative Dermatology, 72*, 1-10.

Umeda, H., Takeuchi, M., & Suyama, K. (2001). Two new elastin cross-links having pyridine skeleton- Implication of ammonia in elastin cross-linking in vivo. *The journal of Biological Chemistry, 276* (16), 12579-12587.

Uytterhaegen, L., Claeys, E., Demeyer, D., Lippens, M., Fiems, L. O., Boucqué, C. Y., Van de Voorde, G., & Bastiaens, A. (1994). Effects of double-muscling on carcass quality, beef tenderness and myofibrillar protein degradation in belgian blue white bulls. *Meat Science, 38*, 255-267.

Valcourt, U., Merle, B., Gineyts, E., Viguet-Carrin, S., Delmas, P. D., & Garnero, P. (2007). Non-enzymatic glycation of bone collagen modifies osteoclastic activity and differentiation. *The Journal of Biological Chemistry, 282* (8), 5691-5703.

Van Assche, G., Van Hemelrijck, A., Rahier, H., & Van Mele, B. (1995). Modulated Differential Scanning Calorimetry: Isothermal curve and virtification of thermosetting systems. *Thermochimica Acta, 268*, 121-142.

Van Hook, A. (1947). Biodynamica, 6, 81 cited in Flory, P. J. & Garrett, R. R. (1958). Phase transition in collagen and gelatine systems. *Journal of American Chemical Society, 80* (18), 4836-4845.

Van Moeseke, W., de Smet, S., Claevs, E., & demever, D. (2001). Very fast chilling of beef: effects on meat quality. *Meat Science, 59* (1), 31-37.

Veis, A. & Cohen, J. (1960). Reversible transformation of gelatin to the collagen structure. *Nature, 186*, 720-721.

Veis, A., & Schlueter, R. J. (1964). The macromolecular organization of dentine matrix collagen, I. characterization of dentine collagen. *Biochemistry, 3* (11), 1650-1657.

Verzijl, N, DeGroot, J., Thorpe, S. R., Bank, R. A., Shaw, J. N., Lyons, T. J., Bijlsma, W. J., Lafeber, P. J. G., Baynes, J. W., & TeKoppele, J. M. (2000). Effect of collagen turnover on the accumulation of advanced glycation end products. *The Journal of Biological Chemistry, 275* (50), 39027-39031.

Verzijl, N., DeGroot, J., Oldehinkel, E., Bank, R. A., Thorpe, S. R., Baynes, J. W., Bayliss, M. T., Bijlsma, J. W. J., Lafeber, P. J. G., & TeKoppele, J. M. (2000). Age-related accumulation of maillard reaction products in human articular cartilage collagen. *Biochemical Journal, 350*, 381-387.

Veit, G. Kobbe, B., Keene, D. R., Paulsson, M., Koch, M., & Wagener, R. (2006). Collagen XXVIII, a novel von Willebrand factor a domain containing protein with many imperfections in the collagenous domain. *Journal of Biological Chemistry, 281*, 3494-3504.

Verzer, F. (1964). Ageing of collagen fiber. *International Review of Connective Tissue Research, 2*, 243-300.

Vitek, M. P., Bhattacharya, K., Glendening, J. M., Stopa, E., Vlassara, H., Bucala, R., Manogue, K., & Cerami, A. (1994). Advanced glycation end products contribute to amyloidosis in alzheimer disease. *Proceedings of the National Academy of Sciences USA, 91*, 4766-4770.

Vlassara, H., Brownlee, M., & Cerami, A. (1985). High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: A potential mechanism for the removal of senescent macromolecules. *Proceedings of the National Academy of Sciences*, 82, 5588-5592.

Vogel, K. G., Paulsson, M., & Heinegård, D. (1984). Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *Journal of Biochemistry, 223*, 587-597.

Voges, K. L., Mason, C. L., Brooks, J. C., Delmore, R. J., Griffin, D. B., Hale, D. S., Henning, W. R., Johnson, D. D., Lorenzen, C. L., Maddock, R. J., Miller, R. K., Morgan, J. B., Baird, B. E., Gwartney, B. L., & Savell, J. W. (2007). National beef tenderness survey-2006: Assessment of Warner-Bratzler shear and sensory panel ratings for beef from US retail and foodservice establishments. *Meat Science, 77,* 357-364.

Von Seggern, D. D., Calkins, C. R., Johnson, D. D., Brickler, J. E., & Gwartney, B. L. (2005). Muscle profiling: Characterizing the muscles of the beef chuck and round. *Meat Science, 71*, 39-51.

Wagner, J. R., & Aňon, M. C., (1985). Denaturation kinetics of myofibrillar proteins in bovine muscle. *Journal of Food Science, 50*, 1547-1563.

Wang, C., Valtavaara, M., & Myllyla, R. (2000). Lack of collagen type specificity for lysyl hydroxylase isoforms. *DNA and Cell Biology*, 19 (2), 71-77.

Wang, X., Bank, R. A., TeKoppele, J. M., & Agrawal, C. M. (2001). The role of collagen in determining bone mechanical properties. *Journal of Orthopaedic Research, 19*, 1021-1026.

Wall, M. S., Deng, X., Torzilli, P. A., Doty, S. B., O'Brien, S. J., & Warren, R. F. (1999). Thermal modification of collagen. *Journal of Shoulder and Elbow Surgery, 8* (4), 339-344.

Wanasundara, P. K. J. P. D., Amarowicz, R., Pegg, R. B., & Shand, P. J. (2002). Preperation and characterization of hydrolyzed proteins from defibrinated bovine plasma. *Journal of Food Science, 67,* 623-630.

Warner, K. F. (1952). Adventures in testing meat for tenderness. *Reciprocal Meat Conference Proceedings,* 5: 156-160.

Warrick, H. M., & Spudich, J. A. (1987). Myosin structure and function in cell motility. *Annual Reviews Cell Biology, 3*, 379-421.

Weaver, A. D., Bowker, B. C., & Gerrard, D. E. (2008). Sarcomere length influences postmortem proteolysis of excised bovine *semitendinosus* muscle. *Journal of Animal Science, 86*, 1925-1932.

Weber, A., & Murray, J. M. (1973). Molecular control mechanisms in muscle contraction. *Physiological Reviews, 53*, 612-673.

Weber, T. K. (1999). Angiotensin II and connective tissue: homeostasis and reciprocal regulation. *Regulatory Peptides, 82*, 1-17.

Welke, R. A., Williams, J. C., Miller, G. J., & Field, R. A. (1986). Effect of cooking method on the texture of epimysial tissue and rancidity in beef roasts. *Journal of Food Science, 51* (4), 1057-1060.

Wess, T. J. (2005). Collagen fibril form and function. *Advances in Protein Chemistry, 70*, 342-374.

Westall, F. C., Scotchler, J., & Robinson, A. B. (1972). The use of propionic acid- hydrochloric acid hydrolysis in Merrifield solid phase peptide synthesis. *Journal of Organic Chemistry, 37* (21), 3363-3365.

Wheeler, T. L., Koohmaraie, M., & Crouse, J. D. (1991). Effects of calcium chloride injection and hot boning on the tenderness of round muscles. *Journal of Animal Science, 69*, 4871-4875.

Wheeler, T. L., Koohmaraie, M., Cundiff, L. V., & Dikeman, M. E. (1994). Effects of cooking and shearing methodology on variation in Warner-Bratzler shear force values in beef. *Journal of Animal Science, 72*, 2325-2330.

Wheeler, T. L., Shackelford, S. D., & Koohmaraie, M. (1996). Sampling, cooking and coring effects on Warner-Bratzler shear force values in beef. *Journal of Animal Science, 74,* 1553-1562.

Wheeler, T. L., Shackelford, S. D., Johnson, L. P., Miller, M. F., Miller, R. K., & Koohmaraie, M. (1997). A comparison of Warner-Bratzler shear force assessment within and among institutions. *Journal of Animal Science, 75*, 2423-2432.

Wheeler, T. L., Shackelford, S. D., & Koohmaraie, M. (1997). Standardizing collection and interpretation of Warner-Bratzler shear force and sensory tenderness data. *Proceedings of Reciprocal meat Conference, 50*, 68-77.

Wheeler, T. L., Koohmaraie, M., & Shackelford, S. D. (1997). Effect of postmortem injection time and postinjection aging time on the calcium-activated tenderization process in beef. *Journal of Animal Science, 75*, 2652-2660.

Wheeler, T. L., Shackelford, S. D., & Koohmaraie, M. (1999). Tenderness classification of beef: III. Effect of the interaction between end point temperature and tenderness on Warner-Bratzler shear force of beef *longissimus*. *Journal of Animal Science, 77*, 400-407.

Wheeler, T. L., Shackelford, S. D., & Koohmaraie, M. (2000). Relationship of beef longissimus tenderness classes to tenderness of gluteus medius, *semimembranosus* and *biceps femoris*. *Journal of Animal Science, 78*, 2856-2861.

Wheeler, T. l., Vote, D., Leheska, J. M., Shackelford, S. D., Belk, K. E., Wulf, D. M., Gwartney, B. L., & Koohmaraie. (2002). The efficiency of three objective systems for identifying beef cuts that can be guaranteed tender. *Journal of Animal Science, 80*, 3315-3327.

Whiting, R. C., & Strange, E. D. (1990). Effects of lactic acid on epimysial connective tissues of muscles used for restructured beef steaks. *Journal of Food Science, 55* (3), 859-860.

Wier, C. E. (1949). Rate of shrinkage of tendon collagen - heat, entropy, and free energy of activation of the shrinkage of untreated tendon. Effect of acid, salt, pickle and tannage on the activation of tendon collagen. *Journal of American Leather Chemists Association, 44*, 108-140.

Will, P. A., Henrickson, R. L., Morrison, R. D., & Odell, G. V. (1979). Effect of electrical stimulation on ATP depletion and sarcomere length in delay-chilled bovine muscle. *Journal of Food Science, 44*, 1646-1648.

Williams, P., Warwick, R., Dyson, M., & Bannister, L. *Gray's Anatomy,* 37th ed., Churchill Livingstone: Edinburgh:, 1989.

Wilson, G. D., Bray, R. W., & Phillips, P. H. (1954). The effect of age and grade on the collagen and elastin content of beef and veal. *Journal of Animal Science, 13*, 826-831.

Wirth, C. J., & Rudert, M. (1996). Techniques of cartilage growth enhancement: a review of the literature. *Arthroscopy, 12* (3), 300-308.

Woessner, J. F. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *Federation of American Societies for Experimental Biology, 5* (8), 2145-2154.

Wöhlisch, E. (1932). Die thermodynamik der wärmeumwandlung des kollagens. Kollagen und gelatine. *Biochem. Ztschr. 247*, 329-337. Cited in Flory, P. J., & Garrett, R. R. (1958). Phase transition in collagen and gelatine systems. *Journal of American Chemical Society, 80* (18), 4836-4845.

Wright, N. T., & Humphrey, J. D. (2002). Denaturation of collagen via heating: An irreversible rate process. *Annual Review of Biomedical Engineering, 4*, 109-128.

Wu, F. Y., Dutson, T. R., & Smith, S. B. (1985). A scanning electron microscopy study of heat-induced alterations in bovine connective tissue. *Journal of Food Science, 50*, 1041-1044.

Wu, Z., Bertram, H. C., Kohler, A., Böcker, U., Ofstad, R., & Andersen, H. J. (2006). Influence of ageing and salting on protein secondary structures and water distribution in uncooked and cooked pork. A combined FT-IR microspectroscopic and ^{1}H NMR relaxometry study. *Journal of Agricultural and Food Chemistry*, 54, 8589-8597.

Wu, Z., Bertram, H. C., Böcker, U., Ofstad, R., & Kohler, A. (2007). Myowater dynamics and protein secondary structural changes as affected by heating rate in three pork qualities: a combined FT-IR microspectroscopic and ^{1}H NMR relaxometry study. *Journal of Agricultural and Food Chemistry*, 55, 3990-3997.

Xiong, Y. L., Mullins, O. E., Stika, J. F., Chen, J., Blanchard, S. P., & Moody, W. G. (2007). Tenderness and oxidative stability of post-mortem muscles from mature cows of various ages. *Meat Science, 77* (1) 105-113.

Yamauchi, M., London, R. E., Guenat, C., Hashimoto, F., & Mechanic, G. L. (1987). Structure and formation of a stable histidine-based trifunctional cross-link in skin collagen. *Journal of Biological Chemistry, 24*, 11428-11434.

Youn, J., & Milner, T. E. (2008). Evaluation of photothermal effects in cartilage using FT-IR spectroscopy. *Lasers in Medical Science, 23*, 229-235.

Young, O.A., & Braggins, T. J. (1993). Tenderness of ovine *semimembranosus*: Is collagen concentration or solubility the critical factor? *Meat Science*, *35*, 213-222.

Yue, D. K., McLennan, S., Delbridge, L., Handelsman, D. J., reeve, T., & Turtle, J. R. (1983). The thermal stability of collagen in diabetes rats: Correlation with severity of diabetes and non-enzymatic glycosylation. *Diabetologia, 24*, 282-285.

Zhang, Z., Li, G., & Shi, B. (2006). Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of Society of Leather Technologies and Chemists, 90*, 23-28.

Zinn, D. W., Gaskins, C. T., Gann, G. L., & Hedrick, H. B. (1970). Beef muscle tenderness as influenced by days on feed, sex, maturity and anatomical location. *Journal of Animal Science, 31*, 307-309.

Ziyatdinov, A. S., Gusev, L. V., Vasilevskaya, V. V., & Khokhlov, A. R. (2006). Analysis of correlations in location of hydrophobic and hydrophyllic monomers in protein sequences. *Doklady Biochemistry and Biophysics, 411*, 361-364.

Zot, A. S., & Potter, J. D. (1987). Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annual Review of Biophysics and Biophysical Chemistry, 16*, 535-559.